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No. 24
THE CONTROL OF LIPID METABOLISM

THE CONTROL OF LIPID METABOLISM

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held at Oxford, 19 July, 1963

ORGANIZED BY
G. POPJÁK

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J. K. GRANT



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INTRODUCTION

By G. POPJÁK

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Biochemistry is an experimental subject; accordingly, our Society, true to the nature of its discipline, is staging today a public experiment in conducting a discussion meeting. It is the first time that communications to be presented at a meeting of the Society have been preprinted *in extenso*. We hope that you have made full use of these preprints and studied them, for it is the intention of the speakers only to draw your attention to the salient features of their papers, and to present any new information obtained since the time the communications were written several months ago. We hope that this type of presentation will stimulate a lively discussion. By the end of the day we shall know whether the experiment staged was good, bad or indifferent.

I wonder whether it is a good omen for our proceedings today that this lecture hall has a very notable historical connection? It was in 1860 that the British Association held a meeting in Oxford, when the famous debate on Darwinism between Bishop Wilberforce and T. H. Huxley took place in this very room. Bishop Wilberforce, at the conclusion of his attack on Darwinism, turned to Huxley and "begged to know, was it through his grandfather or his grandmother that he claimed his descent from a monkey?"* Huxley's exact words in reply are not known, for they were spoken ex tempore, but it is recorded that he gave a "'forcible and eloquent' answer to the scientific part of the Bishop's argument."* His retort to the Bishop's personal invective was equally forceful and dignified: "He was not ashamed to have a monkey for his ancestor; but he would be ashamed to be connected with a man who used great gifts to obscure the truth."*

Probably our discussion today will not be of such historical moment as that famous debate, but let us conduct it in the spirit of scientific truth without personal invectives.

I am certainly looking forward to a stimulating day. The Society is fortunate in having been able to assemble the most powerful catalysts of lipid biochemistry, if I may so label our distinguished visitors from overseas: Professor Sune Bergström from Stockholm; Professor Konrad

* *Life and Letters of Thomas Henry Huxley*; by Leonard Huxley, Macmillan and Co., London, 1900. Vol. I, pp. 183, 184.

Bloch from Harvard; Dr. John Lowenstein from Brandeis; Professor Rodolfo Paoletti from Milan; Dr. Dan Steinberg from Bethesda; and Professor Paul Stumpf from Davis, California. We regret that Professor Feodor Lynen from Munich has not been able to attend in person, but we welcome Dr. Schweizer as his deputy. On behalf of the Society I wish to express to them and to the other speakers our warmest thanks for their willing response to the Society's summons to come and enlighten us.

Many years ago a colleague asked me what I was working on in the laboratory. His response to my answer that I was concerned with lipids was prompt and uncompromising: "Very messy and ill-defined substances—I leave them to you". Our gathering today to discuss the factors controlling lipid metabolism is sufficient proof of the advances made since that glib remark 20 years ago. We do not think, any more, lipids so ill-defined, thanks to the efforts of our chemist colleagues; also, we have reached the stage when we can observe and describe with a fair degree of precision the principal enzymic reactions in the synthesis and degradation of most lipids, although there is still much to be learnt. When we look for the reasons for the rapid advances in our field during the last decade, we may safely attribute it to the abandoning of the concept that an integrated structure of the cell was required for the metabolism of lipids, and to the use of sub-cellular fractions: of isolated mitochondria, microsomes and soluble enzymes in our experiments. We gained much new knowledge from experiments by the new techniques, but this is still not enough. Although at present many of us are still engaged feverishly on disrupting cells and solubilizing particle-bound enzymes, this period of learning from the parts must be followed by an integration of the discovered multitude of reactions of lipids into the carefully controlled economy of the whole cell, of the tissues and of the whole organism.

Of course, the very multiplicity of the enzymic reactions of lipid synthesis and degradation offers many possibilities for control. In the biosynthesis of fatty acids, for example, the principal reactant, acetyl-CoA, may be supplied by the breakdown of carbohydrate, of fat, of amino-acids and by reactions related to the metabolism of these principal cell constituents: 3-hydroxy-3-methyl-glutaryl-CoA by its cleavage gives acetyl-CoA and free acetoacetate, which in turn—after reaction with the succinyl-CoA/acetoacetate transferase—may give two further molecules of acetyl-CoA. The citrate cleavage reaction furnishes also acetyl-CoA, and according to recent work by Lowenstein and his colleagues, may be a very important source of C_2-units in the synthesis of fatty acids in the mammary gland. Thus the rate of fatty acid synthesis may be affected in the first place by the supply of acetyl-CoA. This may sound very simple and obvious to all, but the sites of formation of acetyl-CoA in the cell are not necessarily identical with the sites of fatty acid synthesis. Acetyl-CoA derived from β-oxidation of fatty acids is generated in the mitochondria, whereas fatty acid synthesis is

mostly extra-mitochondrial. Is, then, acetyl-CoA transferred from mitochondria to other compartments of the cell, or is it first cleaved to free acetate, which then diffuses out of mitochondria to be reactivated with CoA and ATP? Is this the role of the notorious acyl-CoA deacylase, which plagues many experiments involving CoA-esters as substrates?

It is also conceivable that the acetyl-CoA generated inside the mitochondria diffuses out in a different form. If the role of the citrate-cleaving enzyme is not confined to an organ of only periodic physiological significance, the mammary gland, but is active in other tissues also, acetyl-CoA may diffuse out of the mitochondria, after condensation with oxaloacetate, in the form of citrate—as was suggested by Lowenstein—and may then be regenerated by the citrate-cleaving enzyme.

Citrate may effect some other controlling influence also in fatty acid synthesis. It has been known ever since the first cell-free enzyme system from chicken liver for fatty acid synthesis was described by Gurin, that citrate and also isocitrate powerfully stimulated the synthesis from acetate, and it was shown in Green's laboratory that this stimulating action was not related to the generation of reduced NADP by isocitric dehydrogenase. Tietz and I have also found that in the soluble enzyme system of the mammary gland free malonate caused an enormous stimulation of fatty acid synthesis from acetate. These effects were unexplained until last year, well after the discovery by Brady, Wakil and Lynen that not only acetyl-CoA, but also malonyl-CoA, formed by the carboxylation of the former by the biotin containing acetyl-CoA carboxylase, was an important reactant in fatty acid synthesis. In the enzyme systems studied, mainly from the liver and mammary gland, the activity of the acetyl-CoA carboxylase—and I choose the word *activity* rather than *concentration* deliberately—was found to be rate-limiting in respect of fatty acid synthesis.

Vagelos and Martin, and Coniglio and I reported, last year at the Society's meeting in Belgium, that the stimulating action of citrate, isocitrate and of malonate on fatty acid synthesis was entirely due to increasing the activity of the acetyl-CoA carboxylase in the enzyme preparations. Vagelos and his colleagues have further shown that this increased activity of the carboxylase was associated with the formation of the trimer of the enzyme. These observations seem to me of much interest. First, they suggest that aggregation of enzyme subunits or conformational changes of enzyme proteins may be of vital importance in their activity, and second, that the levels of intracellular citrate may regulate the activity of acetyl-CoA carboxylase.

Interestingly enough, the activity of the acetyl-CoA carboxylase may not only be rate-limiting in fatty acid synthesis, but it may also have a qualitative effect on the type of fatty acids synthesized. Coniglio and I have observed that the mammary gland enzyme system, when made to synthesize fatty acids from acetate in the presence of CoA, ATP, Mn^{++}, CO_2, and reduced NADP (i.e., when the system had to generate both

acetyl-CoA and malonyl-CoA) synthesized fatty acids from octanoic to hexadecanoic in about equal amounts. However, when the system was provided with preformed acetyl-CoA and malonyl-CoA (the latter in excess of the former), the fatty acid synthesized was almost exclusively hexadecanoic. Perhaps the activity of acetyl-CoA carboxylase determines the concentrations of the short-chain fatty acids in the milk-fat of various species.

The idea of changes in protein conformation causing changes in enzymic activity brings me to another topic, cholesterol biosynthesis. Gould and I proposed first, and Nancy Bucher confirmed and extended the proposition, that the most severely rate-limiting reaction in cholesterol biosynthesis was the reduction of HMG-CoA to mevalonate. Studies started in Lynen's laboratory showed that the level of the HMG-CoA reductase could vary enormously in an organ within a very short time. I cannot believe that an enzyme protein would be destroyed within a day or two of a simple physiological manipulation, such as administration of cholesterol, or caused to be synthesized in an enormous amount within an equally short period of time after administration of a detergent (Triton) or after the treatment with X-rays. I tend to the view that here, too, like with the acetyl-CoA carboxylase, we may have to look for conformational changes of protein in order to account for levels of enzyme activity. May it not be that some of the hormonal effects we shall hear about are also mediated through some such effects?

A Chairman's duty is to be brief, to keep silent most of the time and to allow those who have something really worthwhile to say to have the floor. I, therefore, call upon our esteemed colleague and friend, Professor Konrad Bloch, to report on the biosynthesis of unsaturated fatty acids, a subject in which we still have large gaps in our knowledge.

THE BIOLOGICAL SYNTHESIS OF UNSATURATED FATTY ACIDS

By KONRAD BLOCH

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TRANSFORMATIONS of saturated into unsaturated compounds are common metabolic reactions serving, broadly speaking, two major purposes. In energy production, reduced molecules are dehydrogenated to olefins, yielding hydrogen and electrons for ultimate combination with oxygen by way of intermediary electron carriers. In a second type of olefin-producing reaction, usually irreversible, energy is consumed rather than produced. Reactions in this category are as a rule connected with the biosynthesis of cell constituents in which the double bond is an important structural feature for physiological function.

Olefin-producing reactions are of two main chemical types, dehydrogenations and dehydrations, and examples of either occur in catabolic and biosynthetic reactions. Among the dehydrogenases the enzymes which afford α,β-unsaturated carbonyl compounds by transfer of hydrogen or electrons from substrate to flavoprotein are the best characterized. Succinic dehydrogenase, acyl CoA dehydrogenases, dihydroorotic dehydrogenase and 3-ketosteroid dehydrogenase are examples of this class. These reactions are anaerobic, oxygen functioning only as a secondary electron acceptor. The driving force for the elimination of hydrogen is provided by polarized carbonyl groups of either free carboxylic acids or of thioesters.

The biosynthesis of oleic acid and related monounsaturated fatty acids presents a fundamentally different problem because the double bond is introduced at a great distance from any activating group. That the introduction of isolated or non-conjugated double bonds involves enzymes of the flavoprotein dehydrogenase type seemed *a priori* unlikely. It now appears that the two mechanisms available in biological systems for the formation of non-conjugated olefinic acids are indeed of a special type. In one of these mechanisms a saturated acid is converted to an olefinic acid of the same chain length by oxidative desaturation. In the second, a long-chain unsaturated acid can arise anaerobically by elongation of medium-sized β,γ-enoic acids which in turn are produced by dehydration of β-hydroxy acids. Still another as yet obscure

mechanism seems to exist in plants. It is aerobic but apparently does not involve transformations of a saturated fatty acid of the same chain length.

OXIDATIVE DESATURATION OF LONG-CHAIN FATTY ACIDS

The properties of a particle-bound desaturating system from yeast acting on palmityl CoA or stearyl CoA and affording the corresponding Δ^9-enoyl CoA esters in the presence of oxygen and TPNH have been described in some detail (Bloomfield & Bloch, 1958). Oxygen appears to be the primary electron acceptor since it is not replaceable by artificial electron acceptors. Since the reaction exhibits the characteristic dual requirement for TPNH and oxygen, the fatty acid desaturating system was tentatively classified as an oxygenase-type reaction involving hydroxylation or some other oxygenation of the substrate in the primary step. More direct experimental evidence for this view has not been forthcoming, and in fact various potential intermediates such as 9- or 10-hydroxystearate, 9-ketostearate, 9, 10-*cis*-epoxystearate and 9, 10-dihydroxystearate have proven totally inactive as precursors of oleate in the yeast system. Nevertheless we consider it still possible that oxygenation is a component reaction of oxidative fatty acid desaturation mainly because no other systems are known which simultaneously require molecular oxygen and a reductant without affording a hydroxylated product. The formation of hydroperoxides is certainly a possibility, or alternatively, a direct abstraction of hydrogen from saturated carbon atoms without covalent attachment of oxygen to carbon. Neither of these possibilities is easily tested by experiment.

A noteworthy feature of the desaturating systems is that the double bond always enters at the same position relative to the carboxyl group of the fatty acid but that the total length of the carbon chain to be desaturated is not too critical. The enzymes from yeast and also the more recently studied *M. phlei* system (Fulco & Bloch, 1962) do not distinguish between palmitate and stearate, and judging from the natural occurrence of myristoleate and 9-eicosenoate, acids ranging from C_{14} to C_{20} appear to be susceptible substrates for the desaturase systems. The entry of the double bond at a critical distance from the carboxyl group and the fact that the CoA esters are the substrates suggest perhaps that in the enzyme-substrate complex the thioester grouping is in close proximity to either C_9 or C_{10} and thus in a position to participate in hydrogen labilization by a pseudo-transannular reaction. Since the fatty acid chain is flexible it could form a loop rather than extend linearly along the enzyme surface.

There are exceptions to the 9, 10-specificity of the oxidative desaturation mechanism: in the lipids of *M. phlei* the Δ^{10} isomer is the major hexadecenoic acid. It is formed in intact cells by desaturation of palmitate but not by isomerization of palmitoleate (Scheuerbrandt & Bloch, 1962). Curiously, desaturation of palmityl CoA by the cell-free

system of *M. phlei* produces the Δ^9-isomer exclusively (Fulco & Bloch, unpublished). The two isomeric hexadecenoic acids could still be products of a common intermediate if the initial attack on the saturated carbon chain occurred at carbon atom 10. A further exception to the 9, 10-specificity of long-chain fatty acid desaturation has been noted in *Bacillus megatherium*. In this organism stearate and palmitate are converted to the corresponding 5-monoenoic acids (Fulco, Levy, and Bloch, unpublished).

The desaturating enzymes from *M. phlei*, yeast, and rat liver all catalyze the same overall reaction yet differ markedly in other properties (Table 1). While none of the enzymes is soluble, their particle sizes

Table 1. *Properties of stearate-desaturating systems*

	M. phlei	Yeast	Rat liver
Sedimentation	100,000 × g, 1–2 hr.	17,000 × g, 15 min.	Microsomal fraction
Substrate	stearyl CoA	stearyl CoA	stearate
Pyridine nucleotide	TPNH	TPNH or DPNH	TPNH*
Flavin nucleotide	FAD, FMN	—	—
Metal ion	Fe^{2+}	—	—
Heat-stable cofactor	+	—	—

* only in aged preparations.

cover a broad range as shown by the centrifugal speeds necessary for sedimentation. The fact that the liver and yeast enzymes are associated with a much larger complex than the bacterial enzyme probably explains why in the latter case the cofactor requirements are much more apparent than in the former. Differences in cellular localization of the fatty acid thiokinases probably account for the observation that the fatty acids themselves are adequate substrates in the microsomal liver system, while in yeast and in *M. phlei* the free acids are not active as substrates unless CoA, ATP and a supernatant fraction are added. In yeast and in *M. phlei* the requirement for TPNH is absolute whereas in liver it becomes demonstrable only after freezing and thawing of the microsomes. Finally, only the *M. phlei* system responds to FAD (or FMN) and Fe^{2+}. That these cofactors do not stimulate the desaturation reaction in liver or yeast is presumably due to their tight binding to the microsomal complex.

When *M. phlei* extracts are subjected to high-speed centrifugation, the endogenous FAD or FMN appears to remain largely associated with the supernatant fraction. Since the sedimented desaturase shows essentially no activity in the absence of added flavin nucleotides (Fulco & Bloch, 1962) the coenzymes are evidently less strongly bound to protein in this system than in the majority of FAD-enzymes. The flavin nucleotides are effective at catalytic levels whereas TPNH, as in other oxygenase systems, is consumed in stoichiometric quantities. As pointed out already, FAD does not function as the primary electron acceptor

since it fails to sustain desaturation in the absence of oxygen even when it is present in substrate amounts.

There have been no indications so far that any of the desaturating systems acting on fatty acid acyl CoA consist of more than one catalytic component, nor has it been possible to accumulate intermediates by omission of one or several of the required cofactors. Liver microsomes, after repeated freezing and thawing, produce substantial quantities of a polar derivative of stearate resembling a hydroxystearate in chromatographic behavior (Preiss & Bloch, unpublished), but this metabolite is not a precursor of oleate. It may be a chemical artifact derived from a labile oxygenated intermediate. Polar metabolites produced by liver homogenates from stearate are unrelated to oleate synthesis. They have been identified as 18-hydroxystearate, 17-hydroxystearate and octadecane-1, 18-dioic acid (Preiss & Bloch, unpublished).

Since the discovery of fatty acid desaturation several years ago, progress in the understanding of the reaction mechanism has been slow. Perhaps the more favourable properties of the *M. phlei* desaturase and the recognition of FAD and Fe^{2+} as additional cofactors will facilitate the elucidation of this intriguing reaction.

FORMATION OF POLYUNSATURATED FATTY ACIDS

Oxidative desaturations similar to the transformation of stearyl CoA to oleyl CoA appear to operate also when additional double bonds are introduced into long-chain fatty acids. Ordinarily polydesaturation proceeds from the 9-monoenes by insertion of the second double bond into the homoallylic 12–13 position, but there are two major exceptions: the formation of 6, 9-octadecenoic acid in rats raised on fat-free diets (Fulco & Mead, 1959), and of 5, 11-octadecenoic acid in slime moulds (Davidoff and Korn, 1962). At the enzymatic level the transformation of oleate to linoleate has been shown with a microsomal system from *Torulopsis utilis* (Meyer & Bloch, unpublished) and in intact cells with Tetrahymena species (Erwin & Bloch, 1963), and with castor bean leaves (James, 1962). This particular step in polydesaturation appears to have been lost in metazoan evolution since linoleic acid is essential for vertebrate and invertebrate nutrition. Further extension of the double bond system to all-*cis* non-conjugated trienes can proceed either in the direction of the methyl terminal group (plant pathway, α-linolenic acid) or in the direction of the carboxyl group (animal pathway, γ-linolenate and arachidonate) (see Fig. 1). The enzymatic studies of Stoffel (1961) and of Van Nugteren (1962) with liver microsomes and our own with Torulopsis extracts (Meyer & Bloch, unpublished) have revealed that the general properties of the polydesaturation systems are at least superficially similar to the stearate-oleate transformation. Coenzyme A esters are required as substrates, both oxygen and TPNH are essential components of the reactions, and the enzymes are particle-bound.

Since the double bond systems of the polyunsaturated fatty acids are of the divinylmethane type, one would not expect that any of the already existing centers of unsaturation will materially facilitate the introduction of additional double bonds as they might in the formation of conjugated systems. It seems reasonable therefore that these reactions are oxidative desaturations rather than flavoprotein-linked dehydrogenations.

(1)[a] $CH_3(CH_2)_7CH=CH(CH_2)_7COOH \rightarrow CH_3(CH_2)_4\overset{12}{C}HCH_2\overset{9}{C}HCH=CH(CH_2)_7COOH$
oleate → linoleate

(2)[b] $CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH \rightarrow CH_3(CH_2)_4CH=\overset{12}{C}HCH_2\overset{9}{C}HCH_2\overset{6}{C}H(CH_2)_4COOH$
linoleate → γ-linolenate

(3)[c] $CH_3(CH_2)_4CH=\overset{14}{C}HCH_2\overset{11}{C}HCH_2\overset{8}{C}H(CH_2)_6COOH$
homo-γ-linolenate → arachidonate $CH_3(CH_2)_4CH=\overset{14}{C}HCH_2\overset{11}{C}HCH_2\overset{8}{C}HCH_2\overset{5}{C}H_2(CH_2)_3COOH$

[a] Meyer, F., and Bloch, K., unpublished.
[b] Van Nugteren (1962).
[c] Stoffel (1961).

Fig. 1. Desaturation reactions requiring O_2 and TPNH.

CONVERSION OF CYCLOHEXANE CARBOXYLIC ACID TO BENZOIC ACID

It has long been known that animal tissues convert cyclohexane carboxylic acid to benzoic acid (Bernhard, 1938; Dickens, 1950). More recently Mitoma, Posner & Leonard (1958) located this aromatizing system in liver mitochondria. The reaction has attracted our interest as a model system of possible relevance for the formation of conjugated diene systems. Fractionating extracts of mitochondrial acetone powders we have obtained two enzymes, one catalyzing the dehydrogenation of cyclohexylcarboxyl CoA to 2-cyclohexenylcarboxyl CoA, and the second catalyzing the aromatization of the monoenoate to benzoyl CoA (Babior & Bloch, unpublished) (Fig. 2). A diene intermediate has not so far been

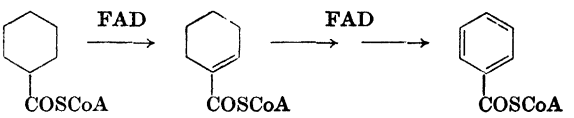


Fig. 2. Dehydrogenation of cyclohexylcarboxyl CoA.

detected. The enzymes are presumably flavoproteins of the acyl CoA dehydrogenase type because both transformations take place under anaerobic conditions and are strongly stimulated by FAD or phenazine methosulfate. Mechanistically, the introduction of the second double bond of a conjugated diene system is comparable to the formation of α,β-unsaturated carbonyl compounds, the olefinic double bond exerting

an activating effect similar to that provided by a polarized carbonyl group.

The most prominent example of conjugated diene synthesis in biological systems is the stepwise transformation of the more saturated carotenoids into the all-*trans* polyenoic carotenes. The enzymes involved have not yet been characterized, but the recent observation of Rilling (1962) that potassium ferricyanide promotes an anaerobic formation of conjugated carotenoids from more saturated precursors certainly points to reactions of the flavoprotein dehydrogenase type. The tentative conclusion seems warranted that separate mechanisms exist for the formation of polyunsaturated systems: anaerobic dehydrogenations for conjugated di- or polyenes and oxidative desaturations for the introduction of methylene-interrupted double bond systems.

ANAEROBIC SYNTHESIS OF UNSATURATED FATTY ACIDS

Monounsaturated fatty acids are universal constituents of cellular lipids, those of anaerobic bacteria being no exception. The known reactions furnishing oleate or palmitoleate in yeast, liver or *M. phlei* are strictly dependent on molecular oxygen as electron acceptor and therefore cannot operate in anaerobic bacteria. Certain distinctive features in the chemistry of bacterial fatty acids also point to the existence of an additional biosynthetic pathway. The work of Hofmann (1952) first disclosed the interesting fact that vaccenic acid (11-octadecenoic acid) and not oleate is the principal monounsaturated fatty acid in Lactobacillus species, and subsequent analytical work has shown that vaccenic acid occurs widely in the lipids of eubacteria (Kaneshiro & Marr, 1962; Scheuerbrandt & Bloch, 1962). The lipids of many Eubacteria are further characterized by greater structural variety of the monounsaturated acids than is commonly found in higher organisms. Thus, two hexadecenoic acids, the Δ^7- and the Δ^9-isomer, and two octadecenoic acids, oleic and vaccenic acids, occur in several Clostridia species. These rather unique structural features suggest that in bacterial systems unsaturated carbon chains can be elongated directly according to the following general process:

$$CH_3(CH_2)_mCH=CH(CH_2)_nCOOH + C_2 \rightarrow CH_3(CH_2)_mCH=CH(CH_2)_{n+2}COOH.$$

A mechanism of this type was first considered by Hofmann *et al.* (1959) on the basis of nutritional studies with lactobacilli; this has been extended and experimentally verified in this laboratory. Retracing the origin of the bacterial octadecenoic acids we were led to conclude that 3-decenoic acid is the earliest olefinic acid which can give rise to vaccenic acid by successive C_2 additions. Correspondingly, 3-dodecenoic acid would be the first unsaturated intermediate in the synthesis of oleate (Scheuerbrandt, Goldfine, Baronowsky & Bloch, 1961). Assuming this mechanism to be correct in principle, the next question to be answered was the origin of the 3-enoic acids and the mechanism by

which they are formed. We have suggested that C_{10} and C_{12}-β-hydroxy acids derived in the conventional way from acetyl CoA and malonyl CoA might undergo β,γ-elimination in addition to the normal α,β-dehydration and thus serve as direct precursors of the 3-enoates. According to the current schemes for the synthesis of long-chain saturated fatty acids (Lynen, 1961), α,β-enoate intermediates are reduced to the corresponding saturated acids whereupon the process of chain elongation is repeated. On the other hand, it seems reasonable to assume that the C_{10} and C_{12} β,γ-enoates which lack the activating influence of adjacent carbonyl groups, resist enzymatic reduction and are elongated as such with the result that the double bonds remain always in the same position relative to the methyl terminal of the fatty acid chain. In this manner successive addition of C_2 units to β,γ-decenoate will yield the series Δ^7-C_{14}, Δ^9-C_{16}, and Δ^{11}-C_{18}. Similar elongation of β,γ-dodecenoate will lead to the Δ^7-C_{16} and Δ^9-C_{18} acids. A general scheme based on these principles, i.e., β,γ-dehydration of β-hydroxy acids and direct chain elongation of 3-enoates as shown in Fig. 3, accounts for all the monounsaturated fatty acids so far encountered in the lipids of Eubacteria. The postulated reactions are also

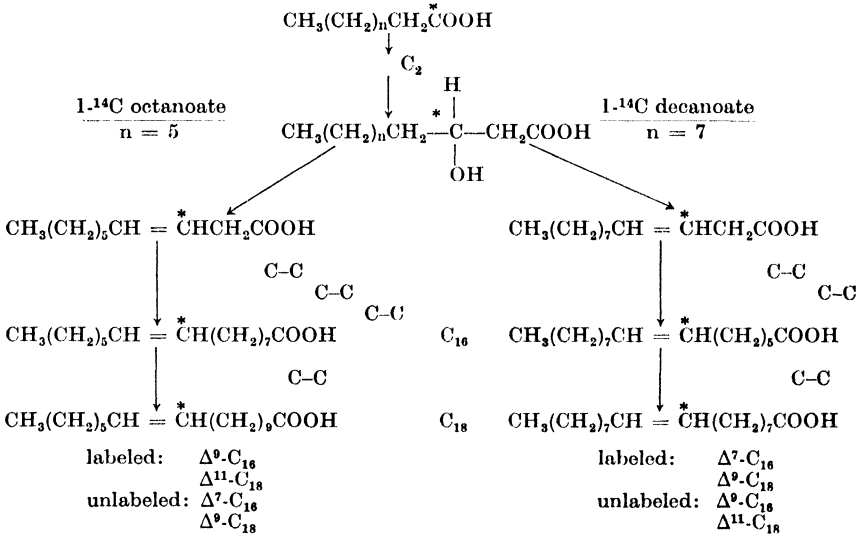


Fig. 3. Bacterial mechanism for the synthesis of fatty acids

consistent with the anaerobic nature of the overall process since the olefinic structure is produced by dehydration rather than by dehydrogenation.

In support of the proposed scheme we have already reported that octanoate and decanoate but not the longer chain acids are converted to C_{16} and C_{18} unsaturated fatty acids by growing cultures of *C. butyricum* (Goldfine & Bloch, 1961). 1-^{14}C-Octanoate afforded one set of isomers (9-hexadecenoate and 11-octadecanoate), and 1-^{14}C-decanoate

the other (7-hexadecenoate and 9-octadecenoate) (Scheuerbrandt, Goldfine, Baronowsky & Bloch, 1961). The prediction that 2-decenoate and 3-decenoate produced from octanoate by way of β-hydroxydecanoate enter separate pathways has been verified as follows (Baronowsky & Bloch, 1962): 1-^{14}C-3-*cis*-decenoate when added to growing cultures of *C. butyricum* yielded labeled C′$_{16}$ and C′$_{18}$ acids but no significant amounts of saturated acids. In the unsaturated fraction only one pair, the 9-C′$_{16}$ and the 11-C′$_{18}$ acids contained ^{14}C in accord with the postulated sequence shown in Fig. 4. When an analogous experiment was

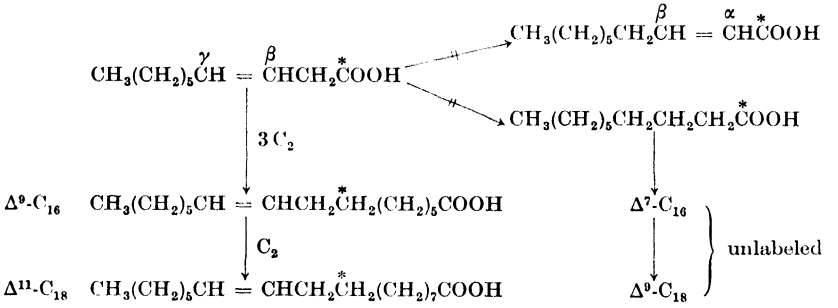


Fig. 4. Metabolism of β,γ-*cis*-decenoic acid.

performed with 1-^{14}C-2-decenoate (*cis* or *trans*) both the saturated and unsaturated fatty acids became labeled. This is consistent with an initial reduction of the 2-decenoate to decanoate and chain elongation to β-hydroxydodecanoate, the latter then yielding 2- and 3-dodecenoate. Since β,γ-dehydration in this case takes place at the C_{12} level, chain elongation by three C_2 units was expected to afford 7-hexadecenoate and thence 9-octadecenoate. This was borne out by the experimental results (Fig. 5).

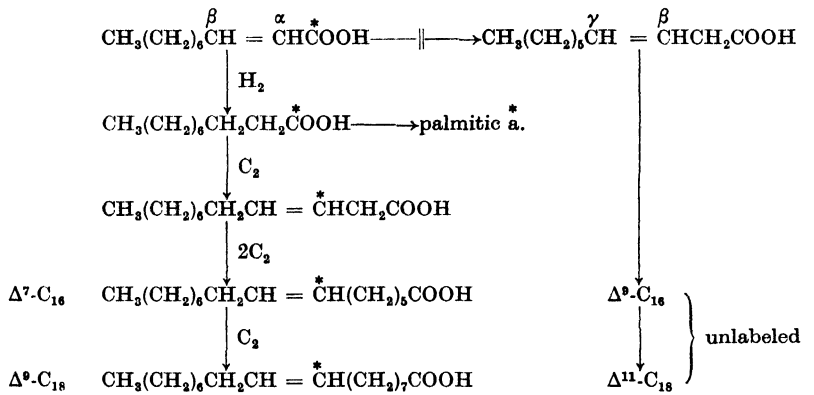


Fig. 5. Metabolism of α, β *cis* decenoic acid

It is of interest that the *cis*- and the *trans*-isomers of 2-decenoate were utilized equally well and gave rise to identical labeling patterns. Whether *cis-trans* isomerization occurred initially or whether the α,β-

reductase is non-specific remains to be ascertained. 3-*trans*-decenoate is apparently not utilized at all.

The respective elongation patterns of the α,β- and β,γ-decenoates in *C. butyricum* are so clearly different that a direct interconversion of the two isomers seems exceedingly unlikely. As indicated earlier, the two decenoates are probably formed separately by dehydration from a β-hydroxy precursor. Attempts to demonstrate dehydration in extracts of *C. butyricum* have failed but these reactions have been successfully demonstrated with a fatty acid synthetase from *E. coli* isolated in this laboratory (Lennarz, Light & Bloch, 1962) and independently by Goldman and Vagelos (1962). The coli enzyme catalyzes the TPNH dependent interaction between acetyl CoA and malonyl CoA much in the same fashion as the liver and yeast palmitate synthetases described by Wakil (1962) and by Lynen (1961). But in contrast to the non-bacterial systems, the E. coli enzyme produces chiefly monounsaturated acids (up to 80% vaccenic acid). The same enzyme complex acts on β-hydroxydecanoyl CoA or the corresponding N-acetylcysteamine derivatives, producing in the absence of any cofactors a mixture of α,β-*trans*- and β,γ-*cis*-decenoates. The isomers are separable by vapour phase chromatography and can be assayed in this manner.

The ratio of the α,β to β,γ forms varies from one enzyme preparation to another and has so far ranged from 4:1 to 1:1. These proportions do not appear to change significantly over a 25-fold range of enzyme purification. We have further observed that in relatively crude preparations [$(NH_4)_2SO_4$ fraction, approximately 5-fold purification], the D- and L-forms of β-hydroxydecanoyl CoA are dehydrated at essentially the same rate and also that they yield the same relative amounts of the isomeric 2- and 3-decenoates (Light & Bloch, unpublished). The dehydrase reaction appears to be reversible since both 2-decenoyl CoA and 3-decenoyl CoA are converted to β-hydroxydecanoyl CoA by the same enzyme preparation. The configuration of these hydration products has not yet been established. It is obviously not possible to decide how many catalytic entities are responsible for these reactions since the enzyme preparation available for the study of the dehydration-hydration reaction is much too impure. The β-hydroxydecanoyl CoA dehydrase from *E. coli* is separable from the fatty acid synthetase system. When purified 35-fold, the dehydrase acts specifically on the D-isomer of β-hydroxydecanoate. Enzyme activity and the composition of the dehydration products (ratio of α, β- to β, γ-decenoates) are strongly influenced by the molarity of phosphate in the incubation medium. High concentrations of phosphate (0·2 to 0.5 molar) stimulate dehydration several-fold and also favour formation of β, γ-decenoate. When combined with the synthetase, the specific β-hydroxydecanoate dehydrase determines the relative proportion of long-chain saturated and unsaturated fatty acids among the reaction products. (Norris & Bloch, 1963).

What we observe, i.e., the apparent lack of specificity for the configuration of the substrate and the simultaneous formation of two positional isomers may be due to the combination of at least two types of activities, one catalyzing racemization or epimerization and another having crotonase-like activity. If the dehydrase reaction is stereospecific, then the epimerization of the D or the L antipode must occur without loss of the hydrogen bound to the β-carbon atom since we have observed no change in $^{3}H/^{14}C$ ratio when 1-^{14}C-3-^{3}H-β-hydroxydecanoyl CoA was the substrate. These data of course rule out a mechanism of epimerization involving the β-keto acids as intermediates. It is also not clear from the available data whether a single enzyme is responsible for the formation of both the α,β- and the β,γ-decenoates. The known properties of enzymes of the crotonase-type do not account for the variety of the reactions we are observing (Table 2). Crotonase catalyzes reversible and stereospecific reactions yielding α,β-*trans*-isomers from L-hydroxy CoA esters and α,β-*cis*-enoates from the D-hydroxy isomers. There is no evidence that crotonase can catalyze a β,γ-elimination reaction.

Table 2. *Reactions catalyzed by* E. coli *dehydrase*

1)	D,L,β-hydroxydecanoyl CoA	→ 3-decenoate + 2-decenoate
2)	D,L-β-hydroxydecanoyl-N-acetylcysteamine	→ 3-decenoate + 2-decenoate
3)	D-β-hydroxydecanoyl CoA	→ 3-decenoate + 2-decenoate
4)	L-β-hydroxydecanoyl CoA	→ 3-decenoate + 2-decenoate
5)	*cis*-3-decenoyl CoA	→ β-hydroxydecanoate*
6)	*trans*-2-decenoyl CoA	→ β-hydroxydecanoate*

* configuration not determined.

Until the dehydrase activity associated with the *coli* synthetase is further resolved, several other questions remain unanswered. For example, the fatty acid synthetase from yeast isolated by Lynen (1961) uses the D-β-hydroxy acid and the *trans*-α,β-enoates as intermediates in the synthesis of palmitate. According to our findings the α,β-*trans*-enoates are intermediates also in *E. coli*, but the data are inconclusive as to the configuration of the β-hydroxy acid precursors. For the same reason it is uncertain whether it is the D- or the L-β-hydroxy acid which gives rise to the β,γ-enoates. These are important points to settle because the composition of the final reaction products, i.e., the relative proportions of long-chain saturated and unsaturated acids will depend *inter alia* on the relative rates at which β-hydroxy intermediates are converted to α,β- and β,γ-isomers. We assume, in the absence of contrary evidence, that in *E. coli* the formation of long-chain saturated and unsaturated acids is catalyzed by the same synthetase and that the presence of β,γ-dehydrase activity is the unique feature which distinguishes bacterial fatty acid synthesis from the analogous (but separate) processes in higher organisms. A more direct test of this hypothesis

should be possible since we have recently been able to separate this dehydrase activity from the *E. coli* fatty acid synthetase by virtue of its greater heat stability (Norris & Bloch, unpublished). The heat-stable enzyme retains the ability to produce a mixture of 2- and 3-decenoates from D,L-β-hydroxydecanoyl CoA.

One other aspect of the bacterial fatty acid synthetase system deserves mention. The β,γ-dehydration of a given medium-chain hydroxy acid appears to occur only once in the course of the overall chain elongation. If this particular step were repeated, di- or polyunsaturated acids with conjugated double bond systems would result. However, acids of this type, or for that matter, any other multiply unsaturated fatty acids are not found in bacterial lipids (Scheuerbrandt & Bloch, 1962). Polyunsaturation apparently occurs only as an oxidative process yielding fatty acids with methylene-interrupted double bond systems.

SYNTHESIS OF MONOUNSATURATED FATTY ACIDS IN GREEN PLANTS

In higher plants the mechanism for the synthesis of oleic acid is distinct from the two pathways discussed so far, at least superficially. Stearate or palmitate desaturation cannot be demonstrated either in the soluble Avocado mesocarp system described by Stumpf (1962) or in growing cultures of *Chlorella pyrenoidosa* (Alter & Bloch, unpublished) or of *Euglena gracilis* (Erwin & Bloch, unpublished). A synthesis of oleate takes place in the extract of Avocado mitochondria described by Stumpf (1962), but curiously only acetyl CoA and not malonyl CoA can provide the C_2 units for condensation. In this particular system chain elongation may well occur by reversal of the mitochondrial fatty acid oxidation, especially since oxygen is required for oleate synthesis. The synthesis of saturated acids is unimpaired under anaerobic conditions, with accumulation of relatively large amounts of stearate as well as of palmitate. These observations are difficult to reconcile with a mechanism of direct desaturation even if it is assumed that some endogenously-formed derivative of stearate rather than stearyl CoA serves as the precursor of oleate. Our studies with *Chlorella* on oleate synthesis from various fatty

Table 3. *Relative specific activity (counts per minute per unit area) of fatty acids*

From C. pyrenoidosa *grown with 1-*^{14}*C labeled fatty acids*

Fatty acid isolated	^{14}C acid added: *2:0*	*8:0*	*10:0*	*12:0*	*14:0*	*16:0*	*16:1 (9)*	*18:0*	*18:1 (9)*
16:0	9663	77	406	789	1230	704	5	18	47
16:1	10958	73	435	470	73	—	940	12	60
18:0	17575	137	681	413	485	134	114	1589	—
18:1	9153	77	243	130	24	12	15	22	1190
18:2	16070	98	481	196	29	25	22	65	856
18:3	8594	32	153	133	23	14	15	71	206

acid precursors have also been inconclusive (Table 3). The medium-chain acids octanoate, decanoate and Δ^3-*cis*-decenoate are efficient precursors of oleate, but these results mean little because there is extensive randomization of isotopic carbon, resulting probably from oxidative breakdown of the precursors. With myristate and palmitate, chain elongation occurred without randomization of ^{14}C, but the extent of conversion to oleate or palmitoleate was insignificant. These particular results, i.e., chain elongation of the C_{14} and C_{16} acids without desaturation of the elongation products strongly speak against the possibility that some endogenous form of stearate differing from stearyl CoA is desaturated to oleate. Stearate itself gave insignificant amounts of labeled oleate. Of all the acids tested, only laurate appeared to be utilized for oleate synthesis in a more direct manner. In oleate derived from 1-^{14}C-laurate the specific activity relative to that of stearate was very much higher than in the corresponding experiments with myristate and palmitate (Table 3). The results of chemical degradation, showing essentially no randomization of ^{14}C, also indicated that oleate had been formed by a relatively direct route from the C_{12} precursor. However, the data shed no light on the mechanism of the desaturation step, nor do they indicate the stage at which the double bond is introduced. A speculative sequence of reactions accommodating the results obtained with *Chlorella* but lacking any other experimental basis can be written as follows:

(1) laurate $\rightarrow$ 2-dodecenoate
(2) 2-dodecenoate $\rightarrow$ β-hydroxydodecanoate $\rightarrow$ 3-dodecenoate
(3) 3-dodecenoate + 3 C_2 $\rightarrow$ oleate.

Reaction 1 would be a component step of mitochondrial fatty acid oxidation; this might explain the oxygen requirement for oleate synthesis observed in the enzyme system from Avocado. Step 2, the isomerization of 2-dodecenoate to the 3-isomer could be direct or it could proceed by way of the β-hydroxy acid in analogy to the β,γ-dehydration step in bacterial systems. The ability of *C. butyricum* to elongate 3-enoates would provide the precedent for step 3, the addition of three C_2 units to 3-dodecenoate to form oleate. As formulated, the hypothetical pathway to oleate in green plants is a variant of the bacterial mechanism to the extent that it visualizes the chain elongation of an already unsaturated precursor but with the essential difference that oxygen is involved in some manner in the process of unsaturation.

The further desaturation of oleate to linoleate and linolenate occurs very readily in green plant systems presumably by the oxygen- and TPNH-linked reactions which have been demonstrated in *Torulopsis utilis* and in liver microsomes. It therefore seems remarkable that green plants, in spite of their ability to carry out oxidative desaturation reactions, have evolved a special mechanism for introducing the first double bond into a saturated fatty acid chain. A wider sample of organisms will have to be analyzed before it can be stated that this

special mechanism is characteristic for all green plants. The generalization does certainly not apply to the blue-green alga *Anabaena variabilis*, an organism capable of desaturating stearate to oleate (Lennarz & Bloch, unpublished). However, *Anabaena* belongs to the primitive algae which are believed to be closely related to the actinomycetes and which furthermore lack the organized chloroplast structure characteristic of higher plants. The above generalization may therefore have to be restricted to the more advanced photosynthetic organisms.

COMPARATIVE ASPECTS

The existence of three separate pathways to monounsaturated fatty acids is a striking example of biochemical diversity which deserves some general comments. We have elsewhere discussed the evolutionary implications of this diversity and have stressed the role which oxygen must have played in the evolution of biosynthetic patterns (Goldfine & Bloch, 1963). Thus the formation of monounsaturated fatty acids by obligate anaerobes is necessarily an anaerobic process and its replacement by oxygen-dependent mechanisms can be viewed as an evolutionary step towards greater efficiency. It should be emphasized that the adoption of the aerobic pathway did not go parallel with the evolution from anaerobic to aerobic energy metabolism (fermentation to respiration), since aerobic eubacteria still employ the "primitive" anaerobic route for synthesizing unsaturated fatty acids. However, the aerobic desaturation pathway operates in the morphologically more advanced actinomycetes, and it therefore appears that the changeover from the anaerobic to the aerobic route occurred at some stage during the evolution of the true bacteria to the mycobacteria. It remains to be seen whether these biochemical parameters are of taxonomic value and whether they can be harmonized with the more traditional classifications

Table 4. *Distribution of pathways for the synthesis of unsaturated fatty acids*

I	II	III
Anaerobic, β,γ-Dehydration-chain elongation; soluble	Aerobic Desaturation; microsomal or small particles	Unknown; (mitochondrial?)
*E. coli**	*Corynebacterium sp.**	
*Lactobacilli**	*Mycobacterium phlei**	
*Clostridia**	*Streptomyces venezuelae**	
*Pseudomonas fluorescens**	*Beggiatoa sp.**	
*Rhodopseudomonas spheroides** (light and dark)	*Anabaena variabilis*	
*Azotobacter agilis**	*Sacharomyces cerevisiae*	*Chlorella pyrenoidosa*
*Agrobacterium tumefaciens**	*Penicillium chrysogenum*	Avocado mesocarp
	Torulopsis utilis	Green leaves
	Astasia longa	*Euglena gracilis* (light and dark)
	Tetrahymena sp.	
	Invertebrates (Insects)	
	Vertebrates (Rat liver)	

* Contain no multiply unsaturated fatty acids.

based on morphological characters (see Table 4). A case in point is our recent observation that in a Corynebacterium species palmitoleate arises by desaturation from palmitate as it does in mycobacteria. One may then raise the question whether this finding is a valid argument against classifying the Corynebacteria among the true bacteria.

The properties distinguishing the aerobic and anaerobic pathways to unsaturated fatty acids are not confined to differences in chemical mechanism. In *E. coli*, for example, the enzymes are present in the soluble portion of the cytoplasm as components of the same complex which catalyzes the general process of chain elongation. In *M. phlei*, in yeast and in liver the desaturation systems are localized in organized cytoplasmic structures and they operate independently from fatty acid synthesis *per se*. Finally, in green plants the synthesis of oleate, whatever its mechanism, appears to be associated with the mitochondria and may involve one or more components of the fatty acid oxidation system. It should be of interest to pursue the correlation between the chemistry of a given pathway and its intracellular localization and to see whether it reflects the parallel development of biochemical and morphological characters.

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DISCUSSION

G. Popják: In the ω-oxidation of stearate with microsomes have you observed any special cofactor requirements and also what is the fate of the ω-oxidized stearate? Will it undergo β-oxidation from both ends or is it going to be oxidized only one carbon at a time.

K. Bloch: We have not found any cofactor requirements for ω-oxidation. The free fatty acid is very actively oxidized and no cofactors are needed. With respect to the further fate of these ω-oxidation products, I can cite only Professor Bergström's experience with model substances, such as α,α'-dimethylstearic acid, which is oxidized to an acid derivative which indicates that the long chains can in fact be oxidized, not necessarily by β-oxidation, but that they can be oxidized from the ω end by shortening of the chain. We have no information as yet as to the fate of the octadecanedioc acid.

P. K. Stumpf: Is TPN required in this system?

K. Bloch: I should think it would be. It is conceivable that after thorough washing the microsomes a TPNH requirement may become apparent.

P. K. Stumpf: In earlier work of others on the shorter chain fatty acids I believe TPNH was an essential cofactor.

K. Bloch: I think it has been demonstrated in the shorter chain acids that this is a TPNH, oxygen, oxygenase type of reaction. Under our experimental conditions we have not been able to demonstrate the need for DPNH.

A. T. James: Did you find any trace of other hydroxy acids? We also found these hydroxy acids occurring in enzyme systems, converting stearate to oleate, but our results suggest a whole string of hydroxy acids with the hydroxy group in many positions in the molecule.

K. Bloch: Apart from the two hydroxy acids which I mentioned, namely 18- and 17-hydroxystearate, and the corresponding derivatives from oleate and palmitate and shorter chain fatty acids, all of which have been degraded and converted into compounds of known structure, we have not found any hydroxy acids carrying an oxygen substituent in a different position.

E. Schweizer: With your desaturating system from yeast have you any evidence that the desaturation occurs in an enzyme bound state in your assay? If this is the case maybe one could explain why one cannot start with an intermediate when this intermediate would not be transferred to the enzyme. We also have observed such an effect with the saturated fatty acid synthesis in yeast. We have evidence that β-hydroxy and β-keto acids are real intermediates but we cannot study the reaction with these compounds or their coenzyme A derivates as substrates.

K. Bloch: We have only indirect evidence on this point. 9- or 10-hydroxy stearate can be activated by intact yeast to form ethyl acetoxystearate and also glycerides, yet it is not converted to oleate.

G. Popják: With the β-hydroxyacyl-coenzyme A dehydrogenase, which seems to be specific for chains of 10 carbon atoms, is there any evidence of isomerase action between α, β which is trans and the β, γ isomers?

K. Bloch: So far we have had no evidence whatsoever that the two isomers are interconvertible. The dehydration reaction is reversible, but we have been unable to show the direct conversion of one double bond isomer into the other.

G. Popják: In the effect of phosphate on the activity of your enzymes, which decides whether they will produce unsaturated in a higher proportion than saturated fatty acids, is it the concentration of phosphate which is critical, or is it merely a question of ion effect?

K. Bloch: From our limited studies it appears that it is a specific phosphate effect. Chloride, sulphate and cations have no effect.

E. Kodicek: Have you any further comment on the stero specificity of your desaturation? I wonder how far Stenhargen's observation, that in 3 dimensions oleic acid appears almost completely flat in 2 dimensions with a kink in the third, has a bearing on this matter. During desaturation could it be that the kink fits into a kink in the enzyme molecule?

K. Bloch: This possibility certainly exists, but I see no way of approaching it experimentally at the moment.

D. H. Nugteren: You showed that in the desaturation of saturated fatty acids cofactors such as pyrimidines and FAD were required. Could you explain the need for these substances?

K. Bloch: We have no mechanistic explanation for the FAD requirement. The optimal concentrations for FAD or FMN in the *M. plei* systems are of the order of 10^{-6}M indicating that the flavin nucleotides act catalytically in contrast to TPNH. So far it has not been possible to demonstrate an FAD requirement in the corresponding liver or yeast systems. These are microsomal enzymes and they may be more difficult to deplete of cofactors than the bacterial system.

THE BIOSYNTHESIS OF UNSATURATED FATTY ACIDS IN HIGHER PLANTS

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In the past many of the workers studying the biosynthesis of fatty acids in plants have noted a very rapid rate of production of unsaturated fatty acids, frequently outstripping that of the saturated acids. Thus Bernhard, Abisch & Wagner (1957) found that the mould *Phycomyces Blakesleeanus* formed from [1-^{14}C]-acetate during the first day 46% of γ-linolenic acid, whose activity then declined slowly to 13% of the total activity of the fatty acids by the 8th day. The activities of oleic, linoleic, palmitic and stearic acids showed less change during the same period. Simmons & Quackenbush (1954) noted that developing soybean seed pods produced labelled oleic acid more rapidly than any other acid from uniformly labelled sucrose. After two days oleic acid contained 56% of the total activity. These authors considered oleic acid to be the precursor of the other acids. (c.f. Newcomb & Stumpf (1953), and Coppens (1956).) Because of this very rapid formation of unsaturated fatty acids in plant systems it has been widely thought that the mechanism of formation of these acids was essentially different from that occurring in mammalian systems and in micro-organisms.

In my laboratory, studies with both intact and chopped leaves have shown a very similar phenomenon when either labelled acetate or malonate were used as precursors. In Fig. 1 is shown a typical gas radio-chromatogram of the fatty acids synthesized by a leaf from [2-^{14}C]-acetate in 3 hours (James, 1963). The highest labelling is found in oleic acid, with palmitic acid second and only a small proportion of activity in stearic acid, although both oleic and stearic acids are minor components.

A time–specific activity curve of the various fatty acids synthesized in an intact leaf over a period of 3 hours (James, 1962) showed that oleic acid had the highest specific activity only 10 minutes from the time of uptake of acetate. Throughout the whole period the specific activity of this unsaturated acid was greater than that of any of the saturated acids and greater than that of linoleic acid. Presentation of the results

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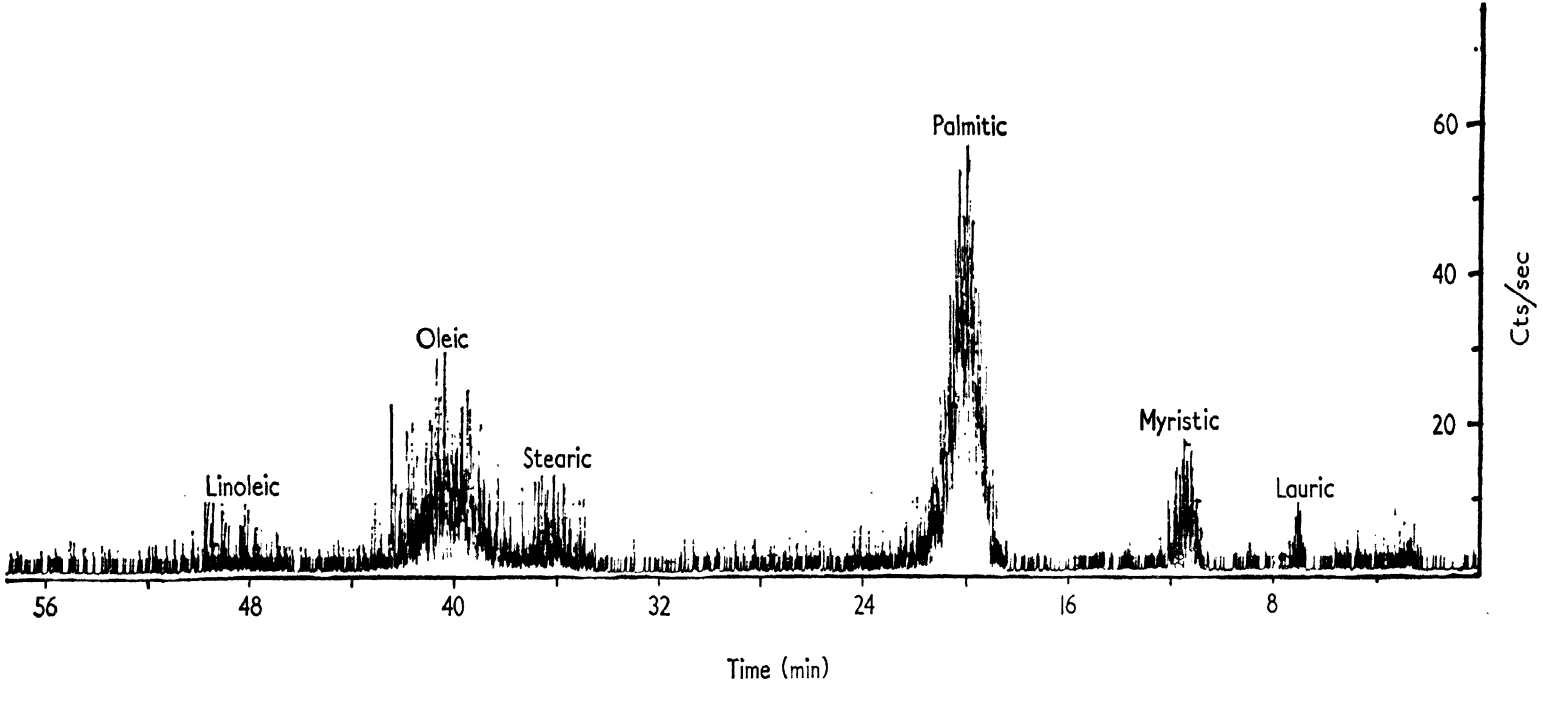


Fig. 1. Labelled fatty acids produced in 3 hours by a chopped lettuce leaf from [2-^{14}C]-acetate, separated on an EGA column at 180°.

in terms of the amount of activity in each acid as a percentage of the total fatty acid activity showed that at no time during the experiment even when the activity of the oleic acid was increasing did there appear to be any but a very small percentage of counts in stearic acid. Such experiments did not show the expected precursor-product relationship for stearic and oleic acids.

Chopped leaves in phosphate buffer are able to take up a variety of longer chain fatty acid substrates. The results obtained from [1-^{14}C]-octanoic, decanoic, dodecanoic, tetradecanoic and octadecanoic acids are shown in Table 1. All except the hexadecanoic and octadecanoic

Table 1. *Specific activity of fatty acids from chopped leaf preparations using different precursors in 0·2 M pH 7·4 phosphate buffer*

Leaf acid	Specific activity in counts/sq. cm. peak area					
	3 hours	5 hours				
	[2-^{14}C]-acetate	[1-^{14}C]-C_{10}	-C_{12}	-C_{14}	-C_{16}	-C_{18}
10:0	0	27,000	0	0	0	0
12:0	0	930	28,600	0	0	0
14:0	630	800	3,900	21,800	0	0
16:0	96	155	216	455	840	0
18:0	low	83	218	354	860	955
18:1	212	205	660	753	0	0
18:2	11·8	34	25	14·5	0	0

acids give rise to labelled oleic acid. The failure of the latter two acids to act as precursors of oleic acid could be explained in a number of ways; viz.

(1) that only the shorter chain acids are readily broken down to acetate and fed back into the synthetic sequence;
(2) only the shorter chain acids are taken up by the tissue, the cell wall being impermeable to the C_{16} and C_{18} acids;
(3) there is a lack of suitable thiokinases capable of converting the C_{16} and C_{18} acids to their CoA esters.

The first explanation cannot be correct for isolation of the labelled oleic acid synthesized from each of the precursors, followed by oxidative degradation and examination of the products on the gas radio-chromatogram, gave the result shown in Table 2. It may be seen that the

Table 2. *Nature of labelled products from oxidative degradation of oleic acid produced from various labelled precursors by chopped leaves*

Precursor	Labelled fragments produced by oxidation of isolated octadecenoic acid	Structure of corresponding octadecenoic acids
(2-^{14}C)-acetic acid	Azelaic, nonanoic acids	Δ^9-octadecenoic acid
(1-^{14}C)-octanoic acid	Nonanoic acid	Δ^9-octadecenoic acid
(1-^{14}C)-decanoic acid	Azelaic acid	Δ^9-octadecenoic acid
(1-^{14}C)-dodecanoic acid	Azelaic acid	Δ^9-octadecenoic acid
(1-^{14}C)-tetradecanoic acid	Palmitic, myristic and azelaic acids	Δ^2-, Δ^4- and Δ^9-octadecanoic acids

labelled fragments (with the exception of those from tetradecanoic acid) are precisely those expected from chain elongation of the precursor without any degradation to acetate.

The second explanation is also incorrect, since both palmitic and stearic acids combined into both galactolipid and phospholipid can be isolated from the leaves. For the same reason explanation (3) also cannot be invoked since presumably formation of galactolipid like that of phospholipid proceeds via and CoA ester.

This evidence would suggest that stearic acid is not a direct precursor of oleic acid in the leaf. The elegant work of Bloch and his group (1962) in demonstrating that the conversion of stearic to oleic acid in cell-free yeast preparations requires molecular oxygen and TPNH has revolutionized our knowledge of unsaturated fatty acid biosynthesis. Bloch *et al.* (1962) have drawn attention to the fact that of the systems they have studied, only those possessing the oxidative pathway, contain polyunsaturated acids. For this reason it would be expected that leaf tissue, whose major fatty acids are linolenic and linoleic, would possess the oxidative pathway of formation of oleic acid. That this is true is demonstrated in Fig. 2, where the results of three parallel incubations of green leaf tissue with [2-^{14}C]-acetate carried out with gas phases of air, argon and argon–5% CO_2 are shown. Similar results obtained with chloroplast free leaf tissue are shown in Table 3. Clearly the lack of oxygen almost completely inhibits the formation of oleic acid and that of linoleic acid, thus the reactions in the leaf are conclusively oxidative. It is, however, interesting that when tetradecanoic acid was used as a precursor the isolated C_{18} monoenoic acid contained a few per cent of the Δ^2- and Δ^4-acids. (In experiments using lettuce leaves it has occasionally been found that appreciable amounts of Δ^{11}-octadecenoic acid are formed.) It would be unwise to assume that the presence of these acids indicates the non-oxidative formation of monoenoic acids by chain elongation of a β-γ unsaturated acid formed by dehydration of the β-OH-acid produced by condensation between a medium chain length acid and acetate, that was recently demonstrated by Bloch and his group (1962) to occur in anaerobic micro-organisms.

The introduction of a double-bond by the oxidative process may not be limited to the 9–10 position. There are many examples of the occurrence of isomeric C_{18} monoenoic acids, e.g., petroselenic acid in parsley seeds. The chemical inertness of the hydrocarbon chain of the long chain saturated acids would suggest that attack upon the chain can occur only after it has been constrained into a particular configuration upon the enzyme surface. Thus no clear assignment of reaction mechanism of the formation of the monoenoic acids can be made simply on the basis of determination of double-bond position.

The failure to get conversion of palmitic or stearic acids to monoenoic acids in leaf tissue is difficult to understand. Either stearic acid is not a precursor of oleic acid, although oxygen is required, or else the enzymes

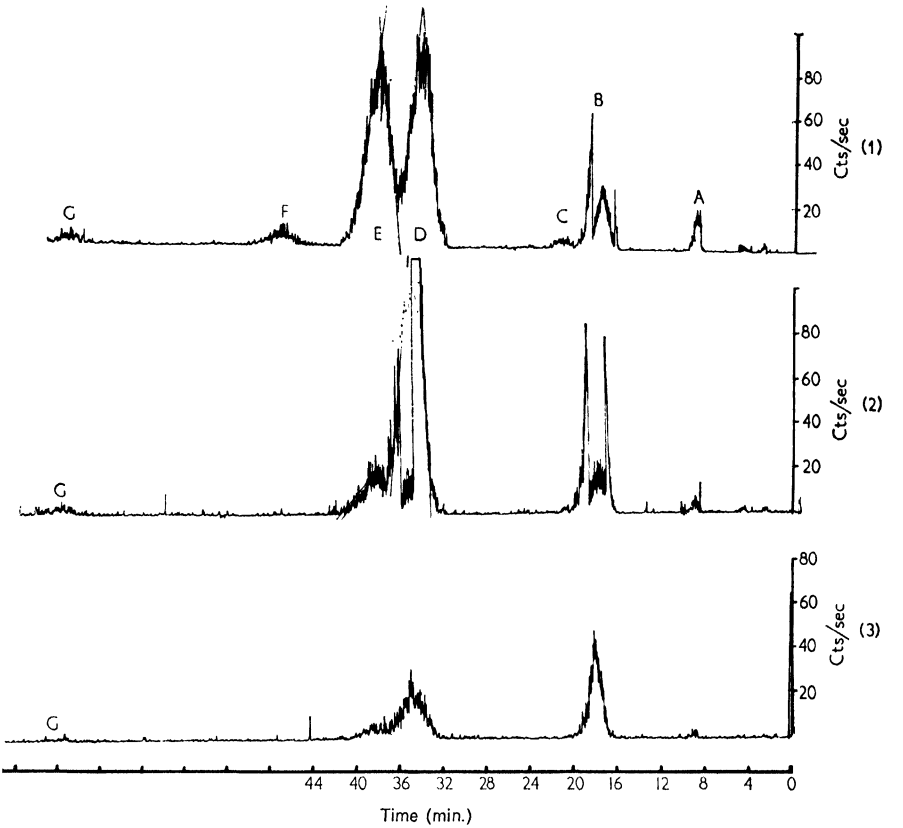


Fig. 2. Comparison of the effects of anaerobic and aerobic conditions on the biosynthesis of oleic acid from [2-^{14}C]-acetate by chopped leaves in phosphate buffer.
Peak identification A, tetradecanoic acid; B, hexadecanoic C, hexadecenoic acid; D, octadecanoic acid; E, Δ^9-octadecenoic acid; F, $\Delta^{9,12}$-octadecadienoic acid; G, eicosanoic acid.

Curve (1) Air as gas phase.
Curve (2) Argon as gas phase.
Curve (3) Argon 5% CO_2 as gas phase.

Table 3. *The effect of air on the biosynthesis of oleic acid in chlorophyll-free chicory leaves using [2-^{14}C]-acetate as precursor*

Fatty acid	Specific activity in counts × 10^3/min./mg. Gas phase – Air	Gas phase – Argon
Lauric	226	226
Myristic	141	178·1
Palmitic	33·8	16·08
Stearic	175·5	143
Oleic	63·5	0
Linoleic	0	0
Linolenic	0	0

necessary for its conversion are located in some part of the cell inaccessible to free palmitic and stearic acid. It is a necessary corollary that the structure should be permeable to the shorter chain acids.

POLYUNSATURATED FATTY ACIDS

The specific activity–time curve referred to earlier suggested a direct conversion of oleic to linoleic acid, as demonstrated by Bloch *et al.* (1962) in a fatty yeast. Leaf tissue is indeed able to convert [1-^{14}C]-oleic acid to labelled linoleic acid in 16% yield in two hours (Table 4). Isolation of

Table 4. *Activity of oleic and linoleic acids isolated from a leaf having [1-^{14}C]-oleic acid as precursor*

Acid	1 hour		2 hours	
	Specific activity counts/sq. cm. peak area in 10 seconds	Total (%)	Specific activity counts/sq. cm. peak area in 10 seconds	Total (%)
Palmitic	—	4·8		2·6
Oleic	—	85·7		81·0
Linoleic	65	9·4	110	16·1

the labelled linoleic acid followed by oxidative degradation and analysis of the products by gas radio-chromatography showed the nonanoic acid portion of the molecule to be unlabelled, whereas azelaic acid was labelled, indicating little if any rearrangement of the labelled carboxyl of the original oleic acid.

Linolenic acid is labelled much more slowly, an appreciable fraction of the total counts turn up in linolenic acid only after 24 hours incubation of leaf tissue with [2-^{14}C]-acetate. The fact that the rise in the linolenic acid count coincides with a fall in the linoleic acid count suggests that the latter is the precursor as suggested by Bloch *et al.* (1962). This point has, however, yet to be strictly proven.

SITE OF FATTY ACID BIOSYNTHESIS IN LEAF TISSUE

Last year in my laboratory Dr Paul Stumpf undertook a study of the particulate fractions of leaf tissue (Stumpf & James, 1963). Careful isolation of the chloroplast fractions showed that these particles were capable of very efficient synthesis of long-chain fatty acids up to oleic acid (see Fig. 3). The chloroplasts, however, did not synthesize more highly unsaturated acids than oleic and also were unable to utilize as substrates the C_8, C_{10}, C_{12} and C_{14} fatty acids, unlike the intact leaf.

The chloroplast itself is particularly rich in lipid (approximately 35% of dry weight). The major components are mono and di-galactolipid, with linolenic acid as the major fatty acid (see Table 5). For this reason it is unlikely that the chloroplast is unable to synthesize the more highly unsaturated acids. The process of isolation may have damaged the

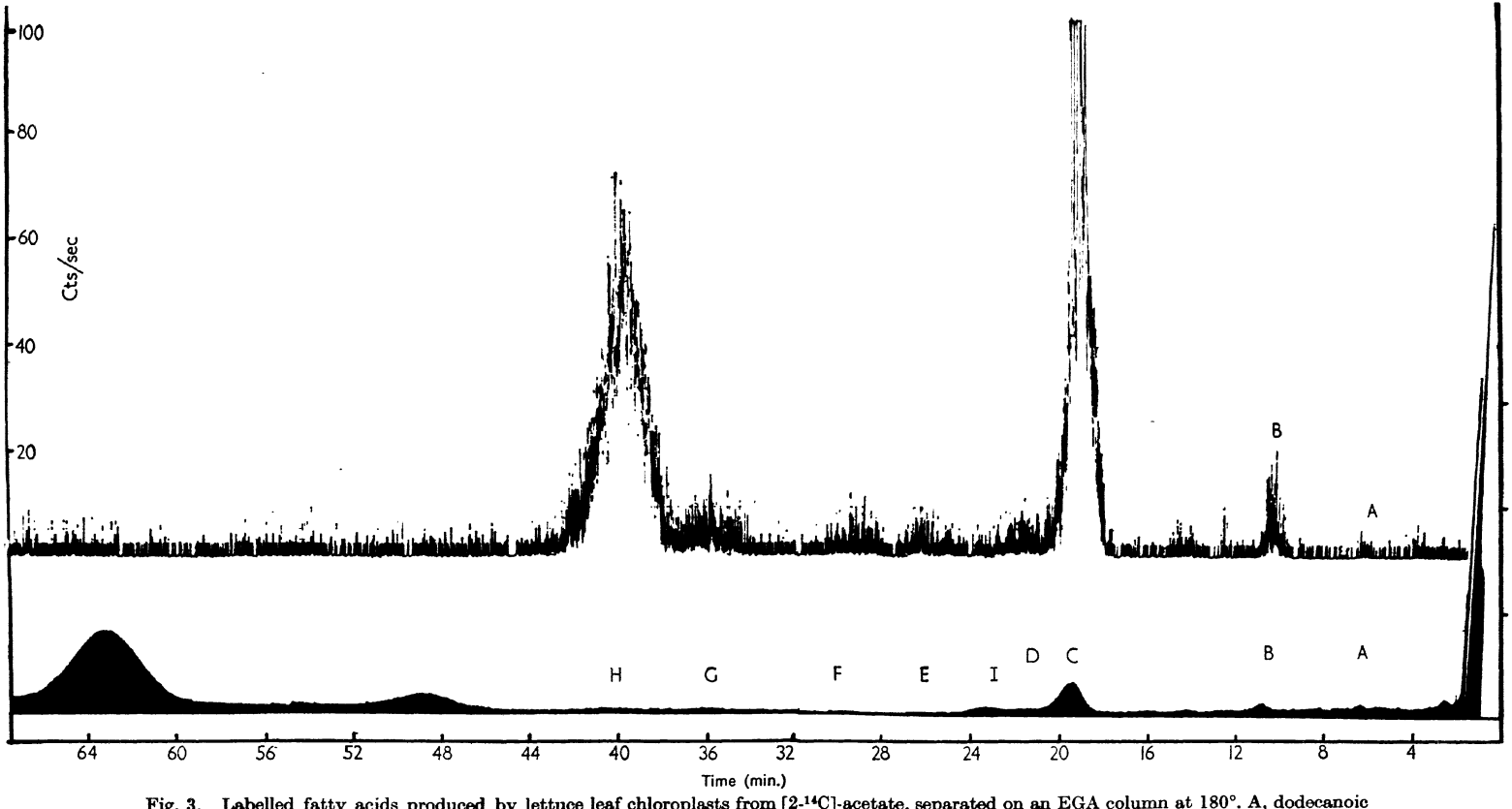


Fig. 3. Labelled fatty acids produced by lettuce leaf chloroplasts from [2-^{14}C]-acetate, separated on an EGA column at 180°. A, dodecanoic acid; B, tetradecanoic acid; C, hexadecanoic acid; D, hexadecenoic acid; E, branched heptadecanoic acid; F, Hepta decanoic acid; G, Octadecanoic acid; H, Δ^9-octadecenoic acid.

Table 5. *Fatty acid composition of lettuce chloroplast lipids as percentage of acids in the range C_8 to C_{20}*

Acid	Monogalactolipid	Digalactolipid	Phospholipids	Undefined neutral lipid
C_8 to C_{10} group	2·1	5·6	3·8	0
12:0	1·1	0·6		1·5
14:0	1·4	1·7	1·2	9·7
16:0	16·2	6·1	7·2	24·6
18:0	3·1	1·8	1·9	6·1
20:0	trace	trace	2·2	trace
17 group	trace	trace	trace	4·4
14:1	0·9	0·8	0·6	3·0
16:1	2·3	1·5	3·8	19·5
18:1	7·3	3·5	3·8	13·2
20:1	1·6	0·9	1·1	0·8
18:2	5·4	5·2	5·4	6·4
18:3	58·5	72·0	69·0	10·6

requisite enzymes since the amount of radio activity in oleic acid increases throughout the whole period of an incubation, whereas in the intact leaf it falls after an initial rise as oleic acid is converted to linoleic acid.

The major points at issue then for plant systems are the following:

(1) Is stearic the direct precursor of oleic acid?

(2) Is the major site of synthesis of the fatty acids of the leaf in the chloroplast? Indeed, are fatty acid and lipid production intimately bound?

NATURE OF THE LIPIDS SYNTHESIZED

The time course of labelling of the fatty acids in the acetone soluble (neutral lipid) and acetone insoluble (phospholipid) showed that both components were turning over at comparable rates. Further separation of the lipid groups by thin layer chromatography disclosed that a small

Table 6. *Lipid components of lettuce leaves. (Nichols private communication (1963))*

Lipid	As percentage of total lipids		
Hydrocarbon	0·2		General class
Sterol esters	0·5		
Triglycerides	2·0	10	Neutral lipid
Free fatty acids	<0·5		
Sterol	5		
Unknown	0·5		
Pigment		20	Pigment
Monogalactolipid	23	43	Galactolipid
Digalactolipid	20		
Phosphatidic acid	2		
Phosphatidyl choline	9		
ethanolamine / serine	4	22	Phospholipid
Glycerol / inositol	4 / <1		
2 unidentified lipids	2		
Sterol glucosides		5	Glucosides

component of the former neutral fraction had a much greater turnover rate (five fold greater than the mono and di-galactolipids and the phospholipids) (see Table 6). The nature of this neutral lipid has yet to be determined.

ADDENDUM

We have now also studied a chloroplast free particulate fraction, isolated by high speed centrifugation, from preparations of leaves. This "mitochondria" like preparation readily synthesizes oleic acid and only oleic acid from acetate but is unable to make either linoleic or linolenic acids. A comparison of the range of acids synthesized from acetate by the three systems is shown in the following table.

	Acids synthesized from labelled acetate		
Tissue	Intact leaf	Chloroplasts	"Mintochondria"
Saturated Acids	C_{10}, C_{12}, C_{14}, C_{16}, C_{18}	C_{10}, C_{12}, C_{14}, C_{15}, C_{16}, C_{17}, C_{18}	None
Unsaturated	Oleic, linoleic linoleic	Oleic	Oleic

The location of the enzyme systems carrying out the conversion, oleic to linoleic and then to linolenic acid is still in doubt.

The direct conversion of oleic to linoleic acid in leaf tissue has now been conclusively demonstrated by Mr. R. Harris in my laboratory. The procedure was to incubate leaf tissue in the light with carboxy labelled oleic acid. Labelled linoleic acid was isolated, reduced to stearic acid and degraded to heptadecanoic acid by the process stearic acid → α-bromo-stearic → α-hydroxystearic acid → heptadecanoic + hexadecanoic + pentadecanoic acids.

Gas radiochromatography of the methyl esters of the mixed acids showed that removal of the terminal carboxyl group caused complete loss of label, hence the linoleic acid was labelled only in the carboxyl position as expected.

It is still a vexed question whether or not oxy acids occur as intermediates in the oxidative introduction of double bonds. Like Dr. Bloch and his group (1963), Dr. J. Marsh and I (1962a, b) were unable to show conversion of a variety of oxystearic acids to oleic acid by either yeast or rat liver preparations. We also demonstrated the occurrence in this system of labelled hydroxystearic acids derived from stearic acid.

Ricinoleic acid (12-hydroxy-Δ^9-octadecenoic acid) bears the same relationship to linoleic acid as 9-hydroxystearic acid to oleic acid. For this reason I have been studying the biosynthesis of this hydroxy-unsaturated acid in immature castor bean embryos. By following the fatty acid composition of the developing bean in the period after flowering it was found that for the first 20–25 days no ricinoleic acid was present. Incubation of [2-^{14}C]-acetate with embryo slices in this period

gave the result shown in Fig. 4—a ready synthesis of palmitoleic, palmitic, stearic and oleic acids with no detectable amount of ricinoleic acid. After 25 days ricinoleic acid suddenly appears and is synthesized

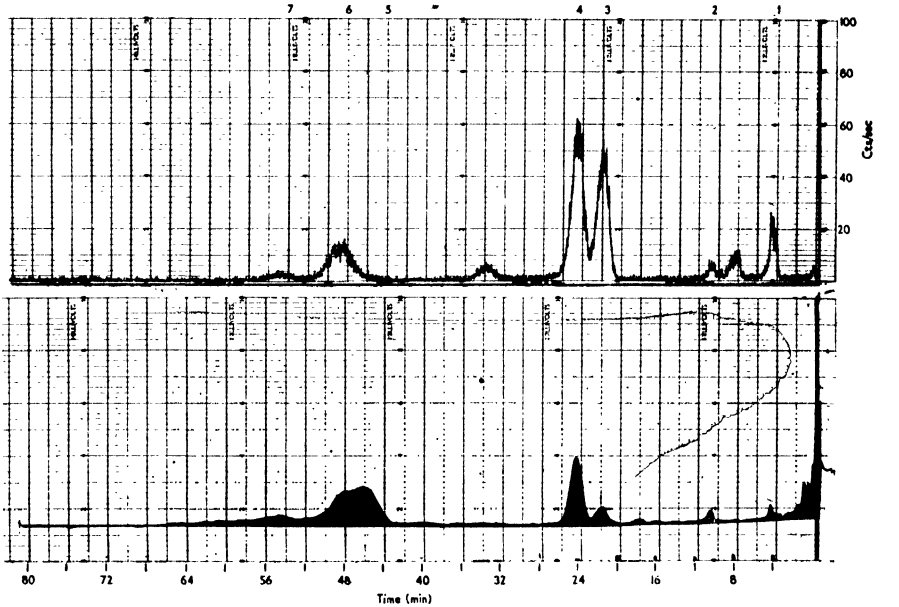


Fig. 4. Radiochemical gas chromatogram of the fatty acids synthesized by the day old castor bean embryo from [2-^{14}C]-acetate in 15 hours. Stationary phase:– Apeizon grease at 200°C. Peaks in order of appearance: (1) n-dodecanoic, (2) n-tetradecanoic, (3) Δ^9-hexadecenoic, (4) n-hexadecanoic, (5) $\Delta^{9,12}$-octadecadienoic, (6) Δ^9-octadecenoic, (7) n-octadecanoic acids.

at such a rate that by 32 days it comprises 80–85% of a vastly increased quantity of fatty acid. During this time slices of embryo were incubated with (a) [U-^{14}C]-linoleic acid or (b) 1-[^{14}C] oleic acid.

In Fig. 5 is shown the result obtained with labelled linoleic acid—no

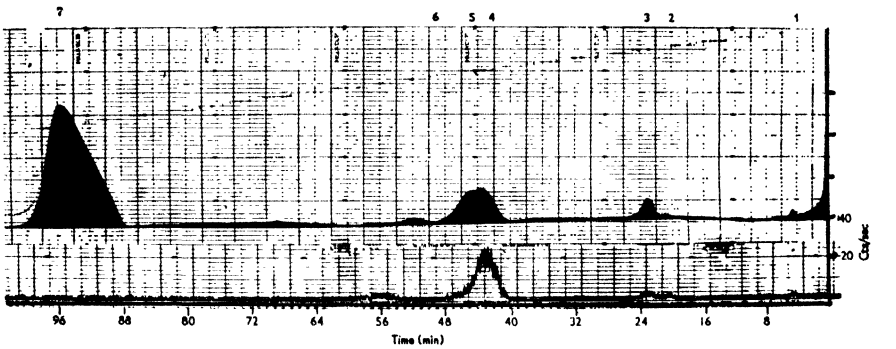


Fig. 5. Radiochemical gas chromatogram of the fatty acids from a mature castor bean incubated with [1-^{14}C]-linoleic acid for 15 hours. Conditions as Fig. 4. Peaks in order of appearance: (1) n-dodecanoic, (2) Δ^9-hexadecenoic, (3) n-hexadecanoic, (4) $\Delta^{9,12}$-octadecadienoic, (5) Δ^9-octadecenoic, (6) n-octadecanoic, (7) ricinoleic acids

conversion to ricinoleic acid could be demonstrated. In Fig. 6 the fatty acids obtained after incubation with [1-^{14}C]-oleic acid show clear labelling in the ricinoleic acid. The yield in different experiments ranging from 22% to 54%. This conversion, unlike the conversion of oleic to

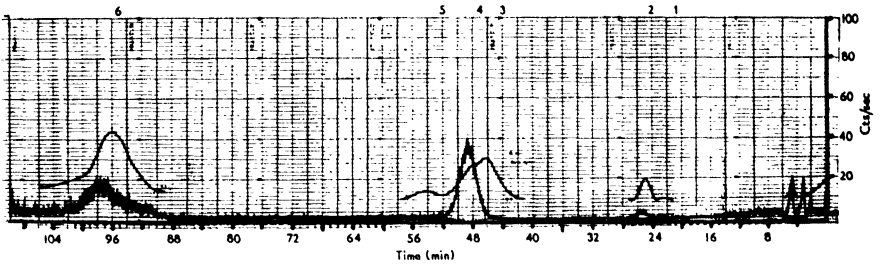


Fig. 6. Radiochemical gas chromatograms of the fatty acids obtained from a mature castor bean embryo after incubation for 15 hours with [1-^{14}C]-oleic acid. Peaks in order of appearance: (1) Δ^9-hexadecenoic, (2) n-hexadecanoic, (3) $\Delta^{9,12}$-octadecadienoic, (4) Δ^9-octadecenoic, (5) n-octadecanoic, (6) ricinoleic acids.

linoleic acid, does not require molecular oxygen—no inhibition being observed under nitrogen. If ricinoleic acid were an intermediate in the conversion of oleic to linoleic acid its formation would require oxygen. This evidence thus rules out ricinoleic acid as such an intermediate.

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DISCUSSION

K. Bloch: I did not quite follow your reasoning excluding linoleic acid as the precursor of ricinoleic acid. There has been a recent demonstration by American workers that oleic acid can be hydrated to 10-hydroxystearic acid by pseudomonads and I think you arrived at the conclusion that there is a direct hydroxylation involved simply from the absence of labelled linoleic acid. Is that correct?

A. T. James: No, if you incubate the embryo system which converts oleic to ricinoleic with universally labelled linoleic acid you get no formation of ricinoleic acid. Hence I think we can rule out the hydration reaction.

G. Goldfine: Have you tested the effect of light on the synthesis of unsaturated fatty acids in the chloroplast under anaerobic conditions? Does light increase the ability of the anaerobic system to produce unsaturated fatty acids?

A. T. James: As far as we can tell the function of light in the chloroplast system is simply to generate ATP and TPNH and there seems to be no other activating effect on the formation of saturated or unsaturated fatty acids. The relative yields are the same in light or dark.

SOME PROBLEMS IN THE CONTROL OF LIPID SYNTHESIS IN HIGHER PLANTS

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INTRODUCTION

In contrast to the fatty acid composition of bacteria, the component fatty acids in higher plants are on the whole quite simple. Palmitic, oleic and linoleic acids are the prominent acids. However, in some plants a fatty acid other than these may be the major component (Table 1).

Table 1. *Unusual fatty acid composition of seed lipids*

Species	*Fatty Acid Composition* (%)						
	14	16	18	18:1	18:2	Unusual Acid	
Hydnocarpus wightiana	—	2	—	6	—	Hydnocarpic	49
						chaulmoogric	27
						gorlic	12
Petroselinum sativum	—	3	—	15	6	petroselinic	76
Ricinus communis	—	—	2	7	3	ricinoleic	87
Picramnia lindeniana	22	33	3	22	—	tariric	20
Verononia anthelmintics	—	3	1·5	2	9	epoxyoleic	78

For example, erucic acid is present in all Cruciferous seed fats; petroselinic acid occurs in seeds of Umbelliferae; chaulmoogric acid dominates in the Flacourtiacae. In some tropical plants such as in the *Palmae*, lauric and myristic acids are main components. In *Ricinus communis*, ricinoleic acid is the overwhelming acid in the matured seed; in *Picramnia* species, tariric acid occurs in great abundance. In general in higher plants, the commonly occurring fatty acids are the C-16 saturated and the C-18 unsaturated fatty acids. But, in addition, there are an important number of plants that have an unusual fatty acid as the major acid.

Leaf lipids contain chiefly the C_{18} unsaturated fatty acids with palmitic acid as the principal saturated fatty acid. Root fatty acids consist mostly of palmitic, oleic and linoleic acids. These tissues possess only small amounts of lipids. It is in the seed lipids where large amounts

accumulate to form the economically important oils. In general, seed lipids are divided into three major groups:

(1) oleic-palmitic group
(2) oleic-linoleic group
(3) linoleic-linolenic group

We have already referred to seed fats which contain as their main component a fatty acid quite different from the commonly occurring acid. In all groups, the composition is remarkably stable and this suggests control mechanisms which are inherently genetic in character.

In recent years studies have been conducted to determine changes in the fatty acid composition in several oil-bearing seeds during the seed-maturation period (Sims, McGregor, Plessers & Mes, 1961). Thus in flax, oleic, linoleic and linolenic acids accumulated very slowly to 10 days after the time of fertilization of the ovule (Table 2). After 10 days

Table 2. *Fatty acid composition of flax and safflower as a function of maturation time of seed* (Sims *et al.*, 1961)

Days after Fertilization	mg./100 seeds							
	Safflower				*Flax*			
	Oil	18:1	18:2	18:3	Oil	18:1	18:2	18:3
10	—	—	—	—	11	4	2	?
20	486	115	298	1·0	148	32	27	61
30	732	151	454	—	244	41	47	110
40	839	141	571	—	272	61	52	118
50	872	149	602	—	—	—	—	—

the rate of formation of oleic and linoleic acids increased whereas linolenic acid formation was much more rapid. In safflower, after 10 days, though the rate of formation of oleic increased noticeably, linoleic acid formation was markedly higher. Linolenic acid is a minor component in this seed oil. Similar studies have been described in the maturation of sunflower seeds lipid (Hopkins & Chisholm, 1961) and avocado fruit lipid (Davenport & Ellis, 1959). In some manner enzyme systems involved in the biosynthesis of specific fatty acids are regulated in their activities so that the final composition of fatty acids in the fully matured seed is that dictated by its genetic makeup. There is good evidence that fatty acids are synthesized *in situ* rather than translocated from sources in other parts of the plant. This is emphasized by the observation that, with the exception of the developing seed, the rest of a Ricinus plant—that is, leaf, stem and root—contains no trace of ricinoleic acid but rather contains the normal component of familiar fatty acids (James, 1962, personal communication).

An interesting approach to the study of stable composition of fatty acids in seed tissue is the work on safflower mutations (Knowles & Osman, 1963). The seed of the commercial variety of safflower contains about 76% linoleic, 16% oleic, 1% stearic and 7% palmitic. Several

mutants have been recently isolated which have more or less a reverse relationship of linoleic and oleic acid concentration. Genetic analysis of these seeds have shown one gene as a controlling factor in the shift of fatty acid pattern. When homozygous, this gene yields seeds with a high oleic acid content; its allele, when homozygous, gave a high linoleic but a low oleic content. Combination of the two genes in a heterozygous plant gave values intermediate between the two. Of interest the concentration of palmitic and stearic acids remained unchanged in these variants (see Table 3).

Table 3. *Fatty acid composition of safflower variants* (Knowles & Osman, 1963)

Type	*Fatty Acid Composition* (%)		
Parents	18:2	18:1	16:0
N–10	76·2	16·1	7·7
57–147	8·7	86·8	4·5
F_1			
57–147 × N–10	55·4	38·9	5·7
N–10 × 57–147	59·4	34·0	6·6
F_2*			
Like N–10	75·2	18·0	6·8
Intermediate	62·3	20·9	6·8
Like 57–147	10·0	84·5	5·5

* In expected Ratio of 1:2:1

BIOSYNTHETIC MECHANISMS IN HIGH PLANTS

Newcomb & Stumpf (1952) studied the incorporation of a number of radioactively labelled substrates by tissue slices of cotyledons of maturing and germinating *Arachis hypogaea*. In both types of tissue radioactive acetate was the most effective substrate incorporated into fatty acids (Table 4).

Table 4. *Comparison of acetate-2-C^{14} incorporation into lipid by slice tissue of maturing and germinating seeds of* Arachis Hypogaea (Newcomb & Stumpf, 1952)

Seed	*Exp. No.*	% *As* $C^{14}O_2$	% *Incorporation of Acetate into Fatty Acids*
Maturing	1	10·5	13·6
	2	13·8	34
	3	6·7	8·4
Germinating	4	11·3	10·5
	5	13·2	4·3
	6	13·7	11·2

In the light of our present knowledge, it is surprising that acetate was incorporated rather effectively into long chain fatty acids by germinating tissue. In germinating tissues, the total lipid content declines rapidly and the energy of oxidation as well as the C_2 units derived from this degradation are utilized to form carbohydrates before the initiation

of the photosynthetic process by the young plant. Several systems are geared to utilize effectively fatty acids and the C_2 units:

(1) the β-oxidation system
(2) the tricarboxylic acid (TCA) cycle
(3) the glyoxylate by-pass system.

Thus the β-oxidation system rapidly converts fatty acids to acetyl CoA and then the glyoxylate system effectively combines acetyl CoA units with glyoxylate to form malic acid. The TCA cycle is operative as evidenced by the formation of $C^{14}O_2$ from acetate-2-C^{14}. It is therefore of some surprise that despite these 3 degradative factors, acetate is still incorporated into long chain fatty acids. It is to be noted, however, that in the maturing seed synthesis is greater than CO_2 production while the opposite result is obtained with germinating tissue. It would be of some interest to re-examine this type of tissue to see what factors regulate the anabolic and catabolic streams of reactions.

In recent years, we have studied rather intensively the system involved in synthesis of fatty acids by avocado mesocarp enzymes. In Table 5 are gathered together data on the composition of fatty acids in

Table 5. *Composition of fatty acids in avocado mesocarp and newly synthesized fatty acids by preparations of avocado mesocarp* (Stumpf, 1962)

Type of Tissue	*Percentage*			
	16	18	18:1	18:2
Avocado Mesocarp	20	1	60	18
Mitochondrial Lipid	15	1	70	15
% incorporation of acetate-C^{14} by various preparations:				
Slice	48	10	31	3
Mitochondria	50	30	20	1
Mitochondria acetone powder extracts	25–40	60–75	0	0
Mitochondria sonicate prep.	14	59	(9+13)*	—

* 13% includes polar long chain fatty acid other than unsaturated acids

avocado mesocarp and the pattern of acetate incorporation by different preparations derived from this tissue. While the normal composition contains mostly the C_{18} unsaturated fatty acids, as soon as the mesocarp tissue is tampered with by the biochemist, the pattern is radically altered. Several enzyme systems have been isolated from this tissue and will be described briefly.

In 1957 Stumpf & Barber described a particulate system which readily incorporated acetate-C^{14} into long chain fatty acids. Degradation studies indicated *de novo* synthesis rather than elongation of existing fatty acids. Palmitic, stearic and oleic acids were the principal acids formed. ATP, CoA, CO_2, Mn^{++} were essential cofactors. The addition of short chain acids such as octanoic and decanoic acids or of long chain acids such as palmitic or stearic acids did not alter the pattern nor indicate any participation in the synthesis of the fatty acids. These acids were, however, activated since they were readily incorporated in the triglyceride pool provided ATP, CoA and Mn^{++} had been added. Mudd

& Stumpf (1962) further observed that the degree of aerobiosis greatly altered the amount of oleic acid which was synthesized. In Table 6 is presented a typical experiment which demonstrates the requirement of oxygen for the formation of oleic acid from acetate. Stearic acid synthesis is greatly modified when oxygen is added or omitted and this suggests a precursor relationship. As already indicated, no conversion of either stearic or stearyl CoA to oleic has been observed with this cell-free system.

Table 6. *Effect of oxygen in distribution of incorporated acetate-C^{14} into long chain fatty acids*

Gas Phase	*Distribution of Label*		
	16	18	18:1
air	53	1	46
helium	61	39	0

In addition to the oxygen factor, the type of initial substrate employed is of prime importance. Barron & Stumpf (1962) have shown that if malonic acid is added to a particulate system, the formation of oleic is completely suppressed and only palmitic and stearic acid are formed. However, if acetate is employed, oleic acid and a "polar" fatty acid are consistently formed.

When acetone powders of this particulate system are prepared, addition of acetyl CoA or malonyl CoA to a water extract of these powders, supplemented with the usual cofactors, results in the *de novo* synthesis of only palmitic and stearic acids. The system is extremely sensitive to avidin and appears to be identical to the soluble fatty acid synthetase system of Wakil (cf. Wakil, 1962). Evidently, the more labile enzymes associated with oleic acid synthesis have been destroyed since no trace of oleic acid is synthesized. However, sonication of the fresh particulate system for very brief periods of time and subsequent high-speed centrifugation yields a turbid extract which can synthesize from acetyl CoA under aerobic conditions 59% stearic acid, 14% palmitic, and 9% oleic acid; the remaining 18% is associated with a long chain polar fatty acid having properties of a hydroxy acid. When malonyl CoA is employed as substrate only palmitic and stearic acid are synthesized.

For the past six years, we have observed that from July to December the particulate system declines sharply in its capacity to synthesize long chain fatty acid from acetate or malonate. This observation is complicated by the shift in the type of avocado variety available at the local markets and by the lack of detailed knowledge of harvesting, storage and handling history of the tissue. Dr S. Yang in our laboratory recently observed that during the fall months while the particles were practically inactive, the supernatant—containing soluble proteins free of microsomes—was completely inactive when acetyl CoA was added as a substrate but when malonic acid was added, considerable synthesis

of palmitic and stearic acids was observed. However, during the months of January to July, particles attain a high capacity to synthesize long chain fatty acids from acetate or malonate. Before any conclusions can be derived from these results, an examination of possible altered permeability or change in fragility of the particulate system must be examined and correlated with the activities of key enzymes in the fatty acid synthetase complex.

With these observations in mind, what can be said about control mechanisms in higher plant systems? We can immediately list several important factors which are intimately involved in this question:

A. We know that germinating seeds geared for catabolic reactions retain the capacity to synthesize fatty acids from acetyl CoA despite the several systems competing for this substrate. There may be some type of compartmentation in germinating seed tissue which separates the synthetase from the catabolic activities.

B. We know that in some systems acetyl CoA carboxylase is a limiting factor for the utilization of acetyl CoA. This is true in the soluble enzyme system of the avocado mesocarp. Hatch & Stumpf (1962) have shown the virtual absence of the carboxylase in root systems of four plants although the enzyme is present in high concentrations in other parts of these plants (leaf and seeds).

C. We have evidence to indicate that the type of substrate, acetyl CoA or malonyl CoA, is a determinant in the type of fatty acid synthesized by plant tissues. Thus acetyl CoA is incorporated into unsaturated and saturated fatty acid whereas malonyl CoA participates in saturated fatty acid synthesis. That an initial substrate in some manner governs the ultimate synthesis of the fatty acids is also indicated by the work of Pierard & Goldman (1963) who observed with enzyme extracts of *Mycobacterium tuberculosis* that although in general the assortment of fatty acids synthesized from acetate or malonate is the same, a major shift occurs in the synthesis of hexacosanoic acid. Thus with acetate and non-labeled $KHCO_3$, 18% of the total incorporation represents hexacosanoic acid; when malonate is added, the synthesis is shifted to 40% and with malonic-C^{14} as the sole substrate, 55% is incorporated into hexacosanoic acid. Similar results have been obtained by Wakil (1962) with mitochondrial systems of animal tissue. Whatever the nature of this shift, it does suggest an alternation of synthetic pathways at some point in the elongation mechanism.

D. In addition the extent of aerobiosis in plant system is of prime importance in determining the extent of unsaturation of newly synthesized fatty acids. This is true not only for lipid synthesis in non-photosynthetic tissue but also in photosynthetic tissue.

E. During the process of maturation, a newly fertilized ovum, usually at the age of three weeks, will suddenly lay down at a high rate its major fatty acid components. This observation, noted with a

significant number of seeds, suggests that at some point in the maturation process the enzyme complex required for fatty acid formation is either completed, synthesized, or activated. Within an additional three weeks the process of synthesizing the total complement of fatty acids is completed and no further changes take place. As our knowledge of lipid synthesis becomes more sophisticated, it would be highly profitable to examine the factors that are controlling the synthesis of lipids in the maturing seed.

Table 7. *Activities, sites, cofactors in lipid metabolism of higher plants* (Stumpf, 1963)

Activity	*Cell fraction*	*Cofactors*	*Plants*
β-oxidation	mitochondria	ATP, CoA, Mn, DPN, TPN	many plants
odd-chain fatty acid oxidation	mitochondria	ATP, CoA, DPN, Mn	many plants
biosynthesis of fatty acids	mitochondria	ATP, CoA, Mn, TPN, Co_2	avocado; chloroplasts
glyoxylate by-pass	mitochondria	Mg	oil seeds
Krebs cycle	mitochondria	ATP, Mn, TCA acids	many plants
glycerol oxidation	mitochondria	ATP, Cyt C, TCA acid	peanut
phospholipid synthesis	microsomes	ATP, Mn^{++}	avocado; peanut
triglyceride synthesis	microsomes	ATP, CoA, Mn	avocado
α-oxidation	microsomes + sol. protein	H_2O_2, DPN	peanut
lipases	soluble protein; lipoidal pellicle	—	many plants
lipoxidase	soluble protein	—	many plants
saturated fatty acid synthesis	soluble protein	ATP, CoA, TPN, Mn, Co_2	avocado

In conclusion, we should list the many reactions found in lipid metabolism of higher plants and indicate their sites and cofactors (Table 7). In Fig. 1 we relate these activities. It is immediately evident that the problem of lipid regulation in plant tissues is a complex one.

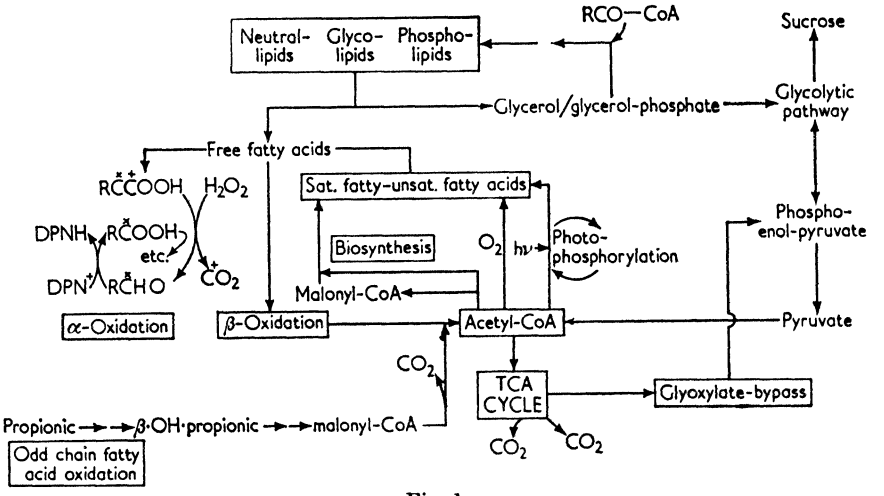


Fig. 1

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DISCUSSION

E. Kodicek: Is it possible that the mechanism of your protein effect is an indirect one due to the protein simply complexing with the fatty acids and preventing them from having an effect?

P. K. Stumpf: That is quite possible but we have added serum albumin and serum globulin to this system and observed no stimulation. Furthermore the protein concentration of this heat stable material is extremely low.

E. Schweizer: Have you any idea about the mechanism of the formation of oleate from acetyl coenzyme A without malonate? Is it the reverse of the degradation or is there some other mechanism?

P. K. Stumpf: I do not think it is the reverse of degradation. We have also added various shorter chain fatty acids to the system for some years and we have always obtained negative results. I think that the anaerobic system and the aerobic system that are described so well in the bacterial system do not apply to the plant systems. We would like to think that the relationship of acetyl coenzyme A to the oleate synthesis is involved somehow in the synthesis of a *de novo* malonyl-coenzyme complex which may be attached to an enzyme protein. When you add malonyl coenzyme A made in the test tube the situation might be quite different from that in the intact enzyme system and would favour *de novo* synthesis of a saturated fatty acid. It seems to me it may be a problem of the formation of a malonyl-sulphydryl enzyme on the enzyme versus the addition of free malonyl coenzyme A to the enzyme and this may be a factor to consider. So far we have never obtained any intermediates between acetate and oleate and we are attacking this from a different point of view now using safflower seedling extracts.

THE REGULATION OF STEROL AND CAROTENOID METABOLISM IN GERMINATING SEEDLINGS

By T. W. GOODWIN and E. I. MERCER

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Ungerminated maize seeds contain sterols but insignificant amounts of carotenoids; when the seeds are germinated in the dark sterols are synthesized and traces of highly oxidized carotenoids appear. If seedlings germinated in the dark for 5–6 days are subsequently illuminated, the morphological changes which take place, as the plastids are converted into chloroplasts, are accompanied by a relatively massive synthesis of carotenoids and of chlorophylls, which contain isoprenoid side chains. There is no corresponding stimulation of steroid synthesis in spite of a marked change in the intracellular distribution of sterol; although this redistribution is associated with chloroplast formation it is not dependent on continuous illumination. The interruption of the dark germination period by a 5 min. exposure to red light (660 mμ) 24–48 hr. before harvesting causes the plastids, normally about the size of mitrochondria, to swell to the size of chloroplasts (Mego & Jagendorf, 1961); this is accompanied by an intracellular redistribution of sterol similar to that observed when 5–6-day etiolated seedlings are continuously illuminated; however, synthesis of plastid carotenoids and chlorophylls does not occur after red light treatment (Cohen & Goodwin, 1961).

If maize seedlings grown for 5–6 days in the dark are excised from their roots, placed in a solution of DL-[2-^{14}C]mevalonate and illuminated for 24 hr., then neither the β-carotene (Goodwin, 1958) nor the phytyl residue of chlorophyll (Mercer & Goodwin, 1962), both of which are rapidly synthesized during this period, is significantly labelled, although it is well established that mevalonate is a specific precursor of terpenoids. On the other hand, activity was found in the phytosterols, in squalene and in a "high counting fraction". The "high counting fraction" has now been separated into three components, two of which have been tentatively identified as β-amyrin and lanosterol; the third component is a Δ^7-stenol which has not yet been identified (Mercer & Goodwin, 1963). All the components of the "high counting fraction" have a much higher specific activity than the β-sitosterol, which is the major sterol of maize. Thus in maize, squalene can cyclize in two ways,

one to form pentacyclic triterpenes such as β-amyrin, and the other to form lanosterol from which the phytosterols are synthesized by an, as yet, unknown route. It is possible that the Δ^7-stenol is an intermediate in the latter biosynthetic pathway.

When the same experiments were carried out in the presence of $^{14}CO_2$ instead of DL-[2-^{14}C]mevalonate, β-carotene and phytol were highly labelled (Goodwin, 1958; Mercer & Goodwin, 1962) whilst no significant activity was observed in the "high counting fraction". The rate of incorporation of $^{14}CO_2$ into the unsaponifiable fraction of the seedlings increases to a maximum over 24 hours, during which period the pigments are being actively synthesized; thereafter pigment synthesis slows down and the CO_2 incorporation falls.

These observations suggest that regulation of sterol, carotenoid and phytol biosynthesis in the early stages of germination is by a process of "compartmentalization". During germination the lipid or carbohydrate reserves of the seed are converted into acetate which is then transformed in the soluble fraction of the cells by the well-known pathway which involves mevalonate and isopentenyl pyrophosphate, to lanosterol and possibly the Δ^7-stenol. Lanosterol or the Δ^7-stenol is then either converted by a soluble enzyme system into phytosterols which are then transported to the plastids, or is transferred to the plastids before conversion into phytosterols. To account for the absence of labelling in β-carotene and phytol in the experiments with [2-^{14}C]-mevalonate, it is assumed that either the substrate is utilized in the cytoplasm before it can reach the plastids, or some substrate does reach the plastids but cannot pass through the cell membrane. On the other hand, $^{14}CO_2$ rapidly penetrates into the developing chloroplast and is equally rapidly incorporated into the plastid pigments which are being actively synthesized. This view would imply that there are two distinct sites of terpenoid biosynthesis within the plant cell, one being in the chloroplast and the other in the cytoplasm.

If the compartmentalization view is correct then the same pattern of incorporation observed with β-carotene and phytol should be observed in the terpenoid side chains of plastoquinone, vitamin K and the tocopherols, which are all compounds specifically located in chloroplasts. The sterol pattern might be observed with ubiquinones, which are extraplastidic, although, as they are located in mitochondria, problems of membrane permeability may also arise. Preliminary experiments indicate that under the conditions described above [^{14}C]mevalonate is not incorporated into plastoquinone whilst $^{14}CO_2$ is actively incorporated.

Plant tissue cultures (Paul's scarlet rose) which do not contain plastids, fit into our general scheme because they synthesize relatively large quantities of sterols, no chlorophylls, only trace amounts of highly oxidized carotenoids and none of the major plastid pigments, β-carotene and lutein (Goodwin & Williams, 1962). The situation with plastoquinone is, however, unexpected; although the levels observed are

lower than those found in green tissues, they are higher than in non-chlorophyllous tissues such as roots. Perhaps even more significant is the fact that the plastoquinone and ubiquinone levels in the tissue cultures are essentially the same.

The unpublished work from this department discussed here has been carried out in collaboration with D. H. Threlfall, W. E. Davies, W. T. Griffiths, K. J. Treharne, B. L. Williams and Ll. Rowlands, and has been supported by the Department of Scientific and Industrial Research and the Air Force Office of Scientific Research, OAR, through the European Office, Aerospace Research, United States Air Force.

ADDENDUM

Subsequent experiments have shown that during chloroplast formation in maize seedlings, [2-^{14}C]mevalonic acid is incorporated into ubiquinone but not plastoquinone, whereas the reverse is true for $^{14}CO_2$ incorporation.

The levels of plastoquinone (PQ) and ubiquinone (UQ_{10}) in the tissue cultures of Paul's scarlet rose (non-photosynthetic meristematic stem tissue) are 0·0368 and 0·0414 μM/g. dry wt. respectively, giving a PQ : UQ ratio of approximately 1 : 1. The presence of plastoquinone in these undifferentiated cultures may be indicative of the presence of plastid primordia, particularly in view of the relative levels of plastoquinone and ubiquinone in etiolated maize shoots (0·0875 and 0·0229 μM/g. dry wt.; PQ : UQ = 4 : 1) and in normal green maize shoots (0·310 and 0·0450 μM/g. dry wt.; PQ : UQ = 7 : 1). The increasing PQ : UQ ratio could be correlated with increasing differentiation and maturity of the chloroplast. Vitamin K_1 and tocopherylquinone, both of which can be readily detected in normal but not etiolated maize shoots, could not be detected in the plant tissue culture.

We visualize the "compartmentalization" of terpenoid biosynthesis within the plant cell as a means by which the cell regulates the production of terpenoid materials to suit its requirements at the various stages of its development. The key to this regulatory mechanism appears to be the impermeability of the plastid membrane to mevalonic acid. Both sites of synthesis (chloroplastidic and extrachloroplastidic) are regarded as having a common biosynthetic "backbone" by which acetate is converted, via mevalonic acid, isopentenyl, geranyl, farnesyl and geranylgeranyl pyrophosphates, through to C_{45} and C_{50} isoprenoid pyrophosphates, presumably by stepwise additions of isopentenyl pyrophosphate. As well as this common biosynthetic sequence, we suggest that each "compartment" has certain specific enzyme systems, the substrates of which are members of the common biosynthetic pathway. Two enzyme systems appear to be characteristic of the extra-chloroplastidic site of synthesis; the first catalyses the tail to tail condensation of two molecules of farnesyl pyrophosphate to produce squalene which is then

cyclized to form sterols and pentacyclic triterpenes; the second system catalyses the incorporation of, presumably, C_{50} isoprenoid pyrophosphate into ubiquinone. The key "backbone" substrate of the chloroplastidic compartment appears to be geranylgeranyl pyrophosphate which is thought to undergo two characteristic reactions. The first of these involves the tail to tail condensation of two molecules to form phytoene, the C_{40} precursor of the carotenoids and in the second, geranygeranyl pyrophosphate is incorporated into the isoprenoid side chains of the chlorophylls, tocopherols and vitamin K_1 presumably via an "active phytol" intermediate. Another reaction characteristic of the chloroplast is considered to be the incorporation of C_{45} isoprenoid (solanesyl) pyrophosphate into plastoquinone.

In summary, therefore, we suggest that the chloroplastidic site of synthesis is responsible for the formation of the chlorophylls, carotenoids, plastoquinone, tocopherols and vitamin K_1 which are all, to a greater or lesser extent, implicated in photosynthesis, whilst the extra-chloroplastidic site synthesizes sterols and pentacyclic triterpenes, whose function is probably structural, and ubiquinone, which is concerned in oxidative phosphorylation.

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DISCUSSION

B. Nichols: I have been working on lettuce chloroplast and find no sterol at all in the chloroplast fraction. Liberal amounts of sterol glycosides occur, however, in the intact leaf but none at all in the chloroplast.

T. W. Goodwin: This is interesting. We have not, however, studied sterol glycosides in any detail. We have only occasionally looked for glycosides in maize, and if they are present in this plant they appear to occur in rather small amounts. Species differences probably account for these observations.

J. Green: We observed some time ago that tocopherol is synthesized to a small but definite extent in maize seedlings germinated in total darkness. I am pleased to know that the synthesis is possible and that the function of the chloroplast is to provide the necessary precursors. If there is enough of the precursors in the germinating seedlings, total synthesis of tocopherol is possible. I should like your view on the observation of Booth at Cambridge that some leaves contain a high proportion of γ

tocopherol and that this occurs outside the chloroplast whereas the other isomers are always inside.

T. W. Goodwin: If you use mevalonic acid apparently it is not incorporated into the tocopherols in darkness. The tocopherol quinone is formed in darkness. We have not considered the differences between the tocopherol isomers to which you refer.

J. Prebble: Have you looked for carotenoid synthesis from mevalonic acid in broken chloroplasts?

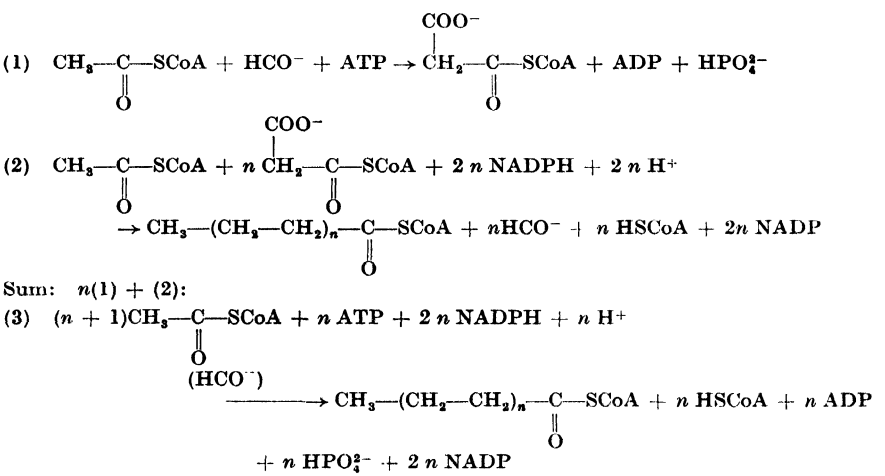
T. W. Goodwin: No, we have looked for carotenoid synthesis from CO_2 in isolated chloroplasts. One of the difficulties is that the incorporation into terpenoids in general stops as soon as the chloroplast is fully matured. So you have to use "semi-mature" chloroplasts which are rather difficult to deal with. We do seem to have a slight incorporation in isolated chloroplasts, but I would not put it any higher than that at the moment. We have not yet tried with broken chloroplasts.

THE CELLULAR CONTROL OF FATTY ACID SYNTHESIS AT THE ENZYMATIC LEVEL

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In the course of our studies on the biosynthesis of fatty acids we were also concerned with the question of the physiological regulation of this process. Of the many influencing factors, which Masoro (1962) has discussed in a comprehensive review, we specialized on the enzymic aspect of the problem. The starting point was the basic work of Drury (1940–41) and of Boxer & Stetten (1944) who observed, in the liver of diabetic or starved animals, a large decrease of the capacity for fatty acid synthesis. This observation has frequently been confirmed (see Langdon & Bloch 1960 for a review). In the search for the cause of this defect, which occurred also in studies on fatty acid synthesis in liver extracts, Numa, Matsuhashi & Lynen (1961) were able to show that in the liver of starved rats the capacity for the formation of malonyl CoA by carboxylation of acetyl CoA (equation 1) was strongly depressed. Wieland, Neufeldt-Eger, Numa & Lynen (1963) were then able to show the same effect in experiments on the liver of diabetic animals.



In all investigated liver extracts, whether from normal, starved or diabetic animals this step appears as the rate limiting one (Numa *et al.*,

1961; Wieland *et al.*, 1963; Ganguly, 1960), since the enzyme for the conversion of malonyl CoA into higher fatty acids (equation 2) is 20 to 100 fold in excess. Therefore this enzymic defect must result in reduced fatty acid synthesis (equation 3). By the addition to the liver extract of purified acetyl CoA carboxylase prepared from yeast it was possible to increase the synthesis of fatty acids by an amount proportional to the activity of the added enzyme. In this manner liver extracts from starved and diabetic animals could be made normal with respect to fatty acid synthesis.

By the comparison of the rates of synthesis in extracts and slices from the same liver, a significant discrepancy was brought to light. Korchak & Masoro (1962) have repeated the experiments on liver extracts from starved animals, and have confirmed our findings. But they also found that, after 24 hours starvation, the acetyl CoA carboxylase activity of the tissue has only sunk to half its normal value, whereas the fatty acid synthesis in liver slices, after the same time, showed only 1–5% of the original rate. Wieland *et al.* (1963) came to a similar conclusion from experiments with diabetic rats in which, after injection of the diabetogenic agent alloxan, they supplied insulin over several weeks and then brought about diabetes by stopping the hormone administration. In the case of the severe ketosis, which was reached 26–27 hours after discontinuing the insulin administration, a strong depression of acetate incorporation into the fatty acids was shown in slice experiments, while the synthesis in liver extracts was only slightly inhibited. Only in animals with long-standing diabetes was the acetyl CoA carboxylase activity so reduced—to around 10% the normal level—that one could correlate it with the existing inhibition of fatty acid synthesis. Therefore, on the basis of all these observations, further factors must be involved in the regulation of fatty acid synthesis in intact liver cells. From experiments aimed at purifying the acetyl CoA carboxylase from liver and studying the properties of the purified enzyme, we arrived at results which could be significant in this connexion.

The starting point for these investigations was a finding, already mentioned (Numa *et al.*, 1961), in our experiments with liver extracts. Measuring the incorporation of acetyl CoA with particle-free rat liver extracts, we showed that, in the absence of citrate or isocitrate addition, there was scarcely any fatty acid formed; whereas, in the synthetic enzyme system containing purified yeast acetyl CoA carboxylase, purified synthetase, and isocitric dehydrogenase, isocitrate could be replaced by $KHCO_3$ and NADPH. We therefore undertook the purification of rat liver acetyl CoA carboxylase, and found that this enzyme, like that from yeast, synthesized malonyl CoA from acetyl CoA, CO_2, and ATP (equation 4), but also required, apart from Mg^{2+} and Mn^{2+}, either citric acid or isocitric acid as cofactor. As is seen from Fig. 1, practically no CO_2-fixation occurs in the absence of citrate. Only after addition of citrate, does the reaction commence. Of interest is the

minute induction phase, which will be discussed in greater detail ›w. By coupling the carboxylase reaction to the pyruvate kinase—

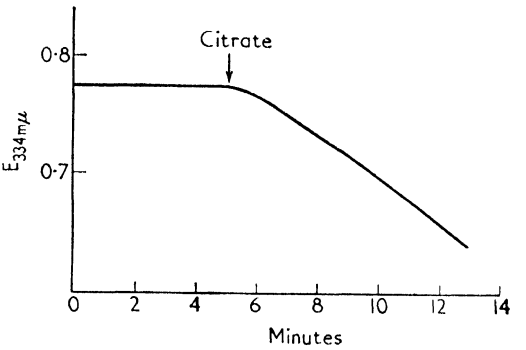


Fig. 1. Dependence of acetyl CoA carboxylation on citrate addition. carboxylase-reaction was coupled with the pyruvate-kinase- and the lactate-dehydrogenase-systems (see equation 4–7).
tions: 40 μMoles K-phosphate pH 7·08, μMoles $MgCl_2$, 0·4 μMoles $MnCl_2$, 20 μMoles O_3 0·15–0·20 μMoles NADH, 0·6 mg serumalbumin, 3 μMoles ATP, 0·10 μMoles l CoA, 0·8 μMoles K-Phosphoenolpyruvate, 10 μg pyruvate-kinase, 25 μg lactate-drogenase, ca. 1 m unit carboxylase. Vol. 0·75 ml. 20 μMoles K-citrate.

ate dehydrogenase system (equations 4–7) it was possible to follow reaction optically at 334 mμ.

$$\text{Acetyl-CoA} + HCO_3^- + \text{ATP} \rightarrow \text{Malonyl CoA} + \text{ADP} + P_i$$
$$\text{Phospho-enol-pyruvate} + \text{ADP} \rightarrow \text{Pyruvate} + \text{ATP}$$
$$\text{Pyruvate} + \text{NADH} + H^+ \rightarrow \text{Lactate} + \text{NAD}$$
: 4 + 5 + 6:
$$\text{Acetyl CoA} + HCO_3^- + \text{Phospho-enol-pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{ATP, } Mg^{2+}} \rightarrow \text{Malonyl CoA} + \text{lactate} + \text{NAD} + P_i$$

he indispensability of citrate or isocitrate for fatty acid synthesis in free liver extracts, as measured by the incorporation of labelled ate, was found by Brady & Gurin (1952). This finding, subsequently atedly confirmed (Langdon (1957), Dituri, Shaw, Warms & Gurin 7); Porter, Wakil, Tietz, Jacob & Gibson (1957) was partly unted for by Brady, Mamoon & Stadtman (1956) as providing DPH, and by Gibson, Titchener & Wakil (1958), as also supplying rbonate, both of which are required for the synthesis of fatty acids a acetyl CoA. The carboxylase preparation, purified by us 340 fold a rat liver homogenates by fractionation with ammonium sulfate, rption on Ca-phosphate-gel, and chromatography on DEAE Cellu-, was free of NADPH- or NADH-oxidizing enzymes, of fatty acid hetase, isocitrate dehydrogenase, and aconitase. These last findings of significance, in that they showed that the mechanism of stimu-n by citric and isocitric acids could in no way be connected with r conversion into oxalosuccinic acid. This again rendered redundant suggestion of Abraham, Matthes & Chaikoff (1960) and Hülsmann

(1960) that the mechanism of the stimulation, by the C_6-tricarboxylic acids, of malonyl CoA formation involved a transcarboxylation between oxalosuccinate and acetyl CoA. Experiments of Numa *et al.* (1961), in which synthetic oxalosuccinic acid was supplied as CO_2-donor, had already excluded this possibility. Oxalosuccinate could not take the place of a combination of ATP, bicarbonate, and isocitrate, in fatty acid synthesis, catalysed by rat liver extracts.

The stimulation of acetyl CoA carboxylase by citrate must thus have other causes. A study of the specificity of the effect could provide a hint in this direction. We tried a series of different acids, for their activation of the carboxylase reaction. The result of this investigation is shown in Table 1. As can be seen, a series of tri- and di-carboxylic

Table 1. *Relative stimulation of acetyl CoA carboxylation by various acids*

	Concentration of acids (μMol/ml)							
	1·0	2·5	6·25	10·0	18·75	25·0	37·5	50
Citrate*	63·7	98·5	91·5	88·0	48·5	35·8		
Isocitrate	30·0	50·0	73·2	86·1	98·1	100·0	87·0	
Malonate		22·1	45·7		84·5			78·9
Fumarate		20·8	43·7		45·8			45·3
cis-aconitate		26	26·0		56·6			
DL-malate			23·5			44·1		
Succinate		7·0		24·4		37·1		49·1
Oxalacetate			13·4					
D-Malate			9·5	14·2		32·0		
β-Ketoglutarate			8·5		18·7			36·7
Trans-aconitate			10·5		23·7			12·2
L-Tartrate						23·1		32·0
Meso-Tartrate						9·7		15·5
Glutarate			3·3		7·2			
α-Ketoglutarate			8·3		4·0			
L-Glutamate					7·2		8·3	
L-Aspartate					4·0		4·5	
D-Tartrate					2·1			4·2
Levulinate			4·5		0·7			
Oxalate					2·5			
Tricarballylate	0·5					4·5		
Maleinate			0·9					

* The stimulating effect of 25 μMole isocitrate /ml is arbitrarily set equal to 100

Optical test in combination with fatty acid synthetase—
Additions: 40 μMoles Tris HCL, pH 7·5, 8 μMoles MgK_2EDTA, 8 μMoles $MgCl_2$, 0·4 μMoles $MnCl_2$, 0·10 μMoles NADPH, 0·6 mg serum albumin, 3 μMoles Glutathione, 20 μMoles $KHCO_3$, 0·10 μMoles acetyl CoA, 10 m units fatty acid synthetase, 0·3–1·5 m units carboxylase; start with 3 μMoles ATP—final vol. 0·77 ml—preincubation time 10 min. at 25°C.

acids were active. Of the mono-carboxylic acids and amino acids, no active compounds have yet been found. The greatest activation-effect was shown by citric, isocitric, and malonic acids. Of these, citric acid showed the highest activity in low concentration range (1–2mM). At 10-times higher concentration, where isocitric and malonic acids gave optimal activation, citric acid was inferior. Nevertheless, the graphical method of Lineweaver & Burk (1934) gave the nearly identical Michaelis Constants: $K_M = 1{\cdot}3 \times 10^{-3}$ Mole Citrate/litre or $1{\cdot}5 \times 10^{-3}$ Mole, D,L-isocitrate/litre.

It should be especially mentioned that D- and L-isocitric acids can tivate to an equal extent, since in a series of experiments with tically active (−)-isocitrate, the same Michaelis Constant $K_M = 1{\cdot}5$ 10^{-3} Mole (−)-isocitrate/litre was found as for that of the racemate. When one studies the list of acids which are activators of the carxylation, and particularly those which cause the activity-increase own by $2{\cdot}5 \times 10^{-2}$ M D,L-isocitrate, one realizes that, without excepn, they are members of citric acid cycle. Yet the very fact that alonic acid is such an effective activator, again confirms that its mulating action is a property of the acid, and not a consequence of metabolic conversion. The same conclusion must be drawn from the tivities of (+)-isocitrate and trans-aconitate, neither of which occur intermediary metabolism. No relationship was found between structre and activating ability. Though citric, malonic and succinic acids ere active, tricarbollylic acid was inactive (Table 1). MgK_2-ethylediaminetetraacetic acid also showed no activating effect, from which may be concluded that the ability to complex with metals is not cisive for the stimulating action.

The study of the mechanism of acetyl CoA carboxylation showed that, analogy with other carboxylation reactions, the rat liver enzyme so contained biotin as the active site. The participation of biotin, in e formation of malonyl CoA from acetyl CoA, had first been shown by akil, Titchener & Gibson (1958) and Wakil & Gibson (1960) in geon liver extracts. With the aid of exchange experiments, which ose out of the studies of Lynen, Knappe, Lorch, Jütting & Ringelann (1959) and Lynen, Knappe, Lorch, Jütting, Ringelmann & chance (1961) on β-methylcrotonyl CoA carboxylase, we were able show that the carboxylation of acetyl CoA also proceeded in two eps:

$$\text{ATP} + \text{HCO}_3^- + \text{Biotinenzyme} \xrightarrow{Mg^{2+}} \text{CO}_2 \sim \text{Biotinenzyme} + \text{ADP} + P_i$$

$$\text{CO}_2 \sim \text{Biotinenzyme} + \text{CH}_3\text{—C(=O)—SCoA} \rightarrow \text{Biotinenzyme} + \text{CH}_2(\text{COO}^-)\text{—C(=O)—SCoA}$$

e formation of carboxybiotin-enzyme from biotin-enzyme, bicarbon-e, and ATP (equation 8), followed by the transfer of the CO_2 from e carboxy-biotin-enzyme to acetyl CoA (equation 9).

In this connexion, the influence of citrate or isocitrate on the separate action steps was of particular interest to us. Table 2 shows the results a series of experiments in which the exchange between [^{32}P]-orthoosphate and ATP was studied. That radioactive ATP was formed en without addition of ATP, is due to the presence of myokinase, in e carboxylase preparation used, which converts ADP to ATP thus:

$$2\,\text{ADP} \rightarrow \text{ATP} + \text{AMP}$$

the experiments shown in Table 2 it was further demonstrated that

the enzyme-bound biotin participated in the catalysis of this exchange-reaction, since it was inhibited by avidin, but was not effected by avidin saturated with (+)-biotin. But what was of greatest interest to us was the demonstration that isocitrate was also required for the [^{32}P]-orthophosphate-ATP exchange to proceed. Taking the measured [^{32}P]-incorporation, in the absence of isocitrate but in presence of avidin, as

Table 2. *ATP⇌^{32}P-exchange*

Incubation mixture	Exchange (mμMole)
Complete (with isocitrate)	15·0
without ADP	0·5
without ATP	4·8
without Mg^{2+}, Mn^{2+}	0·5
+ 0·1 μM acetyl CoA	14·5
+ 150 m units avidin	4·0
+ 150 m units avidin + 2 μg D,L-biotin	14·4
without isocitrate	0·9
without isocitrate, + avidin	0·2
without isocitrate, + avidin + biotin	0·7

Additions: 10 μMoles MgK_2EDTA, 10 μMoles $MgCl_2$, 0·5 μMoles $MnCl_2$, 20 μMoles $KHCO_3$, 0·6 mg serumalbumin, 24 μMoles D,L-K-isocitrate, 0·25 μMoles purified ATP, 0·24 μMoles purified ADP, 33 μMoles [^{32}P]-K-orthophosphate pH 7·5 (6·2 × 10^6 Ipm), 0·033 mg enzyme, vol. 1·0 ml, incubation time 90 min. at 25°C.

zero value, one found that the addition of isocitrate increased the exchange reaction about 20-fold.

The second stage in the carboxylation of acetyl CoA likewise required isocitrate or citrate. This was determined by means of the exchange reaction between [1-^{14}C]-acetyl CoA and malonyl CoA which occurs in presence of the enzyme (equation 9). The result of a typical experiment is given in Table 3. It also shows the involvement of biotin, by the inhibition of the exchange in presence of avidin.

Table 3. [^{14}C]-*acetyl CoA⇌malonyl CoA-exchange*

Incubation mixture	Exchange (mμMole)
Complete (with citrate)	19·1
without malonyl CoA	0·2
without citrate	1·8
complete + 100 m units avidin	2·5
complete + 100 m units avidin + 2 μg D,L-biotin	17·7

Additions: 40 μMoles K-phosphat pH 7·0, 10 μMoles K-EDTA, 0·6 mg serum albumin, 0·10 μMoles [1-^{14}C]-acetyl CoA (105, 600 Ipm), 150 μMoles malonyl CoA, 19 μMoles D,L-K-isocitrate, 0·02 mg enzyme, vol. 1·0 ml. Incubation time, 30 min. at 25°C.

The fact that both exchange-reactions are inhibited by avidin, and require citrate or isocitrate as cofactor, completely excludes the possibility that the C_6-tricarboxylic acids themselves, or their enzymic

ıction products take part in the carboxylation of acetyl CoA as CO_2 ·riers. It might have been supposed that two types of spatially sepa- ɔed active biotin sites were present in animal acetyl CoA carboxylase. these, the one type could have reacted exclusively with ATP and ɔarbonate in the formation of bound CO_2-biotin, while the other biotin ɔe would have selectively catalysed the transfer of the CO_2 from ·boxybiotin-enzyme to acetyl CoA, being unreactive towards ATP d bicarbonate. Were one to suppose that, by their linkage to the pro- n, the two types of active group were not in direct contact with one ɔther, then one could have imagined this spatial separation to be dged by citric acid, or another "activating" carboxylic acid, as inter- ɔdiary CO_2-carrier. Such a conception, which was first discussed by nen (1961) is not, however, consistent with the result of the exchange periments. From this picture it would, indeed, be understandable ıt both exchange reactions, that between [^{32}P]-orthophosphate and ·P as well as that between [1-^{14}C]-acetyl CoA and malonyl CoA, ıuld be inhibited by avidin. But they should be independent of citrate isocitrate since in this case only one of the two biotin sites would be ɔd.

On the same grounds one can exclude a variant of this scheme, dis- ɜsed by us, where two different and spatially separated functional ɔtin groups would be brought into mutual contact by an alteration protein conformation due to a binding of the activating acid to the zyme. An increased folding of the polypeptide chain by addition of rate would be conceivable.

As our concept of different functional reactive groupings, in the acetyl A carboxylase from rat liver, can hardly be maintained in the light the exchange experiments, other representations gain in significance. ey stem from general experience of the structure of the "active site", recently compiled by Koshland (1960) in an excellent review. He agines an "active site" of specific three-dimensional structure, con- ɔted with a definite conformation or tertiary structure of the enzyme- ɔtein; and this model can be directly used to explain the catalytic ture of the activating effect observed on acetyl CoA carboxylase. For ɜre is no reason why only the formation of the specific enzyme- ɔstrate intermediate should be responsible for the induction of the ɜalytically active enzyme conformation from an inactive, or less ɜive, one. In the same way, other substances which bind the enzyme- ɔtein can also bring about a change of conformation and thereby ɜntually influence the catalytic activity in a positive sense. Seen thus, ɜ activation of acetyl CoA carboxylase by citric acid, etc. appears as a ıeral phenomenon in enzyme chemistry, which expresses itself not ly in activation effects, but also—probably more frequently—in in- ɔition effects.

At the outset, one can say little over the ultimate nature of the con- ·mation change brought about by citric acid in the purified acetyl

CoA carboxylase of rat liver. It is a reversible process, since the enzyme, fully activated by citrate or a related anion, loses its high activity again by dialysis, filtration on Sephadex column, or dilution with water, concurrent with the removal or dilution of the citric acid. Furthermore, the reactivation brought about by treating the protein solution with citrate or isocitrate, does not appear immediately, but only after a certain induction time (see Fig. 1). We have studied this appearance in detail.

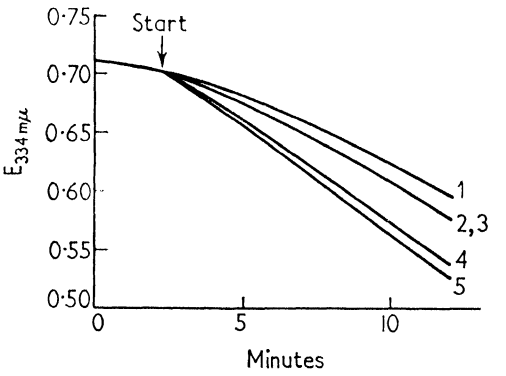


Fig. 2. Effect of preincubation of acetyl CoA carboxylase with citrate or isocitrate on carboxylation kinetics.
Optical test in combination with fatty acid synthetase.
Additions: See Table 1. Start of reaction with: 1. Enzyme; 2. Citrate; 3. Acetyl CoA; 4. HCO_3^-; 5. ATP.

In Fig. 2 a series of experiments is shown, in which malonyl CoA formation is measured optically by its conversion to fatty acids in presence of NADPH and excess fatty acid synthetase. In these, the carboxylation reaction, which required enzyme, acetyl CoA, ATP, bicarbonate, Mg^{2+} and Mn^{2+} as well as isocitrate, was started by the addition of the missing component after 10 min. incubation at 25° of all the other components. If started by $KHCO_3$, ATP or with Mg^{2+}-Mn^{2+}, the reaction proceeded immediately at maximal rate, without induction phase. However, if either enzyme, isocitrate or acetyl CoA was absent during the preincubation, a distinct induction period appeared, which was of about the same duration for each of the three components. In addition, the reaction rate in these cases did not reach the value attained when the carboxylation process was started with bicarbonate. In other experiments, in which citrate was added as activator, we obtained similar results.

From these experiments it may be inferred that for the activation of the enzyme, that is, for the modification of the protein to a favorable conformation, not only isocitrate or citrate but also acetyl CoA is involved. This was surprising since, in studying the exchange between [^{32}P]-orthophosphate and ATP, where acetyl CoA is in no way necessary, we had similarly demonstrated the activation by C_6-tricarboxylic acids. At first we were unable to give an explanation for this difference

in behaviour. However, to ensure that the induction time observed, in the experiments of Fig. 2 with acetyl CoA was not an artefact brought about by the additional optical test system, we also followed the carboxylation reaction in a system without other enzymes, i.e. by measuring the ^{14}C-fixation. The results obtained confirmed the findings from the experiments with added enzyme-test. More $^{14}CO_2$ was fixed after preincubation of the carboxylase together with isocitrate and acetyl CoA, as than with either of the two substances alone.

Vagelos, Alberts & Martin (1962) who have made a detailed study of the citrate activation of acetyl CoA carboxylase from animal fat tissue, similarly came to the conclusion that the activation was due to a change in protein conformation of the enzyme-citrate complex. They found evidence that the activity-increase caused by citrate was paralleled by the formation of a protein aggregate, to give a molecular weight about three times that of the monomer protein which existed in absence of citrate. We have also applied the sucrose-gradient ultracentrifugation method of Vagelos *et al.* (1962) to the purified acetyl CoA carboxylase of rat liver and the result of this experiment is shown in Fig. 3. A sample of the same carboxylase preparation, together with serum albumin as

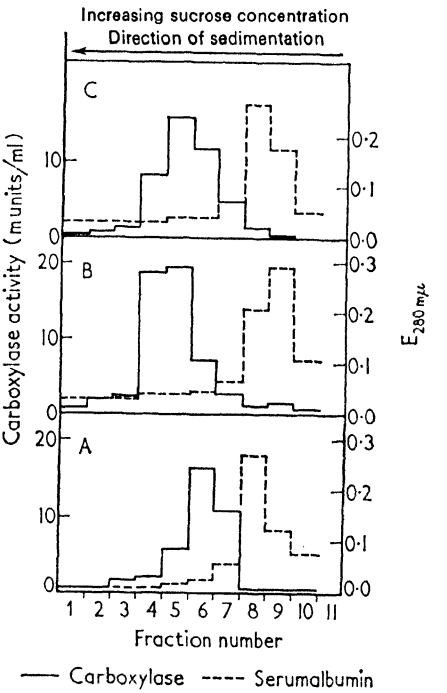


Fig. 3. Ultracentrifugation of activated and unactivated acetyl-CoA carboxylase in a sucrose gradient.

A: Untreated carboxylase.
B: Carboxylase, preincubated with K-citrate (2×10^{-3} M) and acetyl CoA (3×10^{-5} M).
C: Carboxylase, preincubated with K-tricarballylic acid (2×10^{-3} M) and acetyl CoA (3×10^{-5} M).

high-molecular weight reference material, was placed in each of three separate gradient-containing centrifuge tubes in either: 0·01 M phosphate buffer (A), 0·01 M phosphate buffer containing 2×10^{-3} M K-citrate and 3×10^{-5} M acetyl CoA (B), and finally 0·01 M phosphate buffer containing 2×10^{-3} M K-tricarballylic acid and 3×10^{-5} M acetyl CoA (C). The tubes were centrifuged at 39,000 revs./min. for 4 hrs. Fig. 3 shows graphically the distribution of carboxylase activity (continuous line) and protein (dashed line) in the centrifuge tube after centrifugation. As can be seen, the enzyme in contact with citrate and acetyl CoA (B) was more rapidly sedimented than that in only phosphate buffer (A). But since addition of tricarballylic acid as well as acetyl CoA, which does not activate the carboxylase, produces a similar rise in sedimentation rate, we cannot decide on these grounds whether the demonstrated aggregation of the enzyme protein is an essential condition for the activation of the rat liver enzyme. This decision must await further experiments.

The earlier-mentioned findings of Masoro (1962) and Wieland *et al.* (1963) had showed that the inhibition of fatty acid synthesis under certain metabolic conditions, such as diabetes or starvation, could be only partially explained by the loss of acetyl CoA carboxylase in these cells. Considering the amount of inhibition which we found, this assumption did not appear to be fully justified.

The present results, concerning the influence of certain acids of the tricarboxylic acid cycle on the activity of acetyl CoA carboxylase, offer a solution to these difficulties. According to investigations of Frohmann, Orten & Smith (1951) the concentration of these acids is greatly lowered in the liver of alloxan diabetic rats. They showed only about one quarter of the normal value.

According to an interesting theory of Wieland (1961), the deficiency of these cells in citrate, and the acids subsequently formed in the Krebs' cycle, is eventually a consequence of the altered NADH/NAD ratio in diabetic cells as compared to that in normal cells. The strongly increased fatty acid oxidation in diabetes leads to a correspondingly raised NADH/NAD quotient. Among other things this results in a shift of the NADH-dependent equilibrium oxalacetate-malate in favour of malate. Since it is known that in liver oxalacetate represents the limiting component of the citric acid cycle, it is possible that the more negative redox potential of the diabetic cell and the consequent lowering of the oxalacetate level could be the reason for the low citric acid concentration in these cells.

Before we can state with certainty that the acids of the citric acid cycle under physiological conditions play a role in the control of fatty acid synthesis, the distribution of these acids in the cell must be known. To our knowledge this has not yet been determined. A definitive solution to this problem must await the results of further work in this direction.

ADDENDUM

Very recent investigations by Dr. Regen in our laboratory have shown that the citrate level in the rat liver after 24 hr. fasting has sunk to 50–60% of the normal value. Indeed these findings could possibly explain qualitatively the decrease of fatty acid synthesis in such animals based upon the citrate effect. However, the decrease in fatty acid synthesis is much greater than the decrease in the citrate level, which makes it uncertain how important citrate is in the control of fatty acid synthesis.

More firmly based is our information on another regulation mechanism. It has been known for long that fatty acid synthesis is almost fully blocked in starving or diabetic animals. A common characteristic of both situations is the raised level of fatty acids in the blood, and the increased flux of fatty acids in the organs. Further it could be shown in normal animals that a fatty diet or the infusion of chylomicrons lead to a drastic inhibition of fatty acid synthesis. These observations are in accord with the concept of a homeostatic control of fatty acid synthesis. In pursuing this idea, Dr. Bortz in our laboratory recently investigated the effect of higher fatty acids and their CoA derivatives on the purified acetyl CoA carboxylase of rat liver. When the free fatty acids were added to the acetyl-CoA carboxylase mixture, no effect was noted. However, upon the addition of the CoA derivatives a striking decrease in the measured activity of the carboxylase was seen. This decrease appeared to be related to the amount of acetyl-CoA in the incubation mixture and when Lineweaver-Burk plots were constructed for enzyme activities over the range of acetyl CoA concentrations of 5-40 $\times$ 10^{-6}M, in the absence and in the presence of a constant level of long chain acyl CoA, convergent lines were obtained which intersected at the ordinate

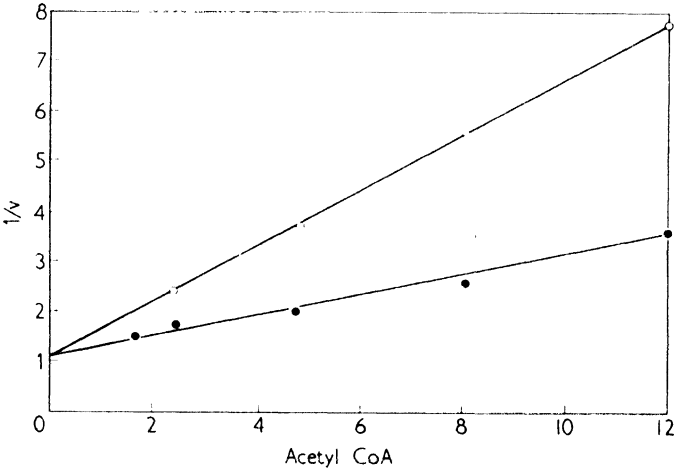


Fig. 4. Lineweaver-Burk plot for acetyl CoA in the presence (○) and absence (●) of $1{\cdot}2 \times 10^{-5}$M palmityl CoA. V is expressed as units optical density change, and acetyl CoA is expressed in molar concentration.

(Fig. 4). This therefore indicated an inhibition of the competitive type. From such plots can be derived the K_I-values of the various acyl CoA derivatives tested. The degree of inhibition is related to the chain length of the added acyl CoA.

Table 4. K values of acyl CoA derivatives

	K_M	K_I
Acetyl CoA	$1{\cdot}9 \times 10^{-5}$	—
Capryl CoA	—	$1{\cdot}4 \times 10^{-4}$
Lauryl CoA	—	$7{\cdot}4 \times 10^{-5}$
Myristyl CoA	—	$1{\cdot}1 \times 10^{-5}$
Palmityl CoA	—	$7{\cdot}2 \times 10^{-6}$
Margarinyl CoA	—	$4{\cdot}3 \times 10^{-6}$
Stearyl CoA	—	$7{\cdot}1 \times 10^{-7}$
Oleyl CoA	—	$1{\cdot}3 \times 10^{-6}$

Here we have a typical case of "endproduct inhibition". As can by seen from a glance at the pattern of lipogenesis (Fig. 5) these inhibitore long-chain acyl CoA derivatives represent the last small molecules in the synthetic sequence, before incorporation into the "complex lipids"

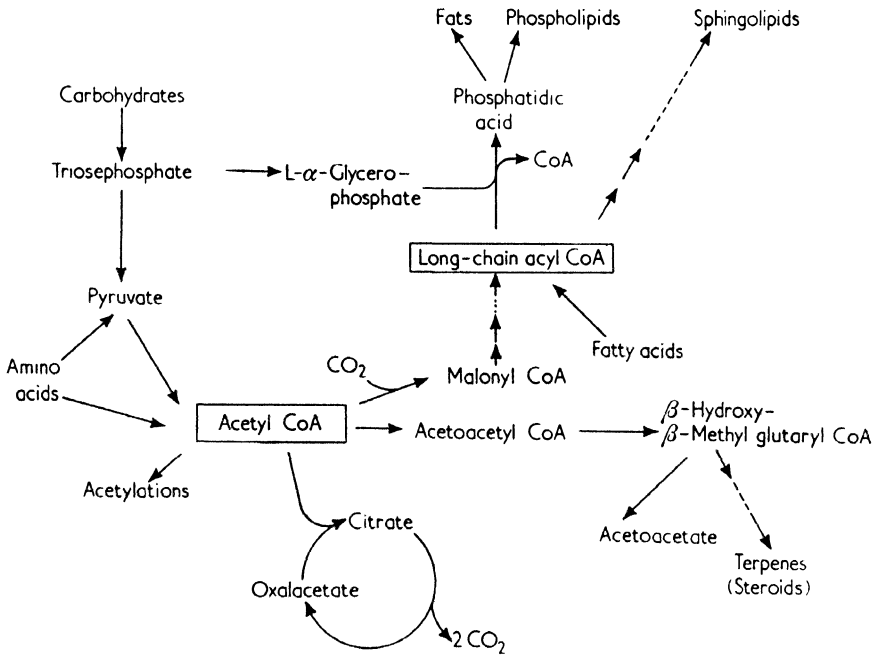


Fig. 5.

occurs. Furthermore, this is a case of "feedback inhibition" of the biosynthetic chain, since the acetyl CoA carboxylase is that point, at which fatty acid synthesis branches off from the many other reaction paths of acetyl CoA.

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DISCUSSION

G. Popják: The activation of the acetyl coenzyme A carboxylase by members of the citric acid cycle and some other acids is remarkable. For example, the activation caused by malonate is extraordinarily specific. Methyl malonic acid is totally inactive. Hence there must be some structural relation between these acids and the conformation of the enzyme protein. You mentioned that the effect of citrate and isocitrate can be reversed by dialysis. Have you observed any effect on the stability of acetyl coenzyme A carboxylase when it is kept with or without citrate or isocitrate? I ask this because, with the acetyl coenzyme A carboxylase from mammary gland, we have found that malonate or citrate is highly effective in preserving the enzyme activity for a very long time in the deep freeze. In the absence of these acids the enzyme is inactivated within ten days.

E. Schweizer: In my experience the purified enzyme remains active for a long time without citrate.

H. Gutfreund: Is the end product inhibition by palmityl coenzyme A or other coenzyme A derivatives related to the aggregation phenomenon which occurs in the presence of citrate or malonate? If so it would be a feed-back inhibition at several steps removed, similar to that demonstrated recently in a number of interesting alosteric phenomena.

E. Schweizer: We have not as yet investigated the aggregation phenomenon in this connection.

R. Dils: We find that albumin strongly stimulates the mammary gland enzyme system which synthesizes fatty acids from acetate and that it is almost impossible to get enough albumin into the incubation mixture to obtain a concentration curve in order to define the maximum effective concentration of albumin. We assume that this effect of albumin is due to the removal of the end products of the synthesis but we are not sure. Have you observed this effect?

E. Schweizer: We did not investigate this same effect but we found that albumin bound fatty acids did not influence the carboxylase. Do you conclude that the function of albumin is to bind the fatty acids?

R. Dils: Yes.

G. Popják: The function of albumin would be to remove the end product, that is fatty acid formed. If you add albumin saturated with some other fatty acid, you might not get the same effect.

E. Schweizer: I agree.

G. Popják: I think these observations on the effect of fatty acids or rather the coenzyme A esters on the activity of the carboxylase are extremely interesting and may represent a very important part in the control of fatty acid synthesis. There are many conditions particularly fasting, diabetes, and so on, in which fatty acid synthesis from acetate is very severely depressed, and these are all conditions in which there is a high concentration of free fatty acids, at least in the blood, or a high concentration of albumin bound fatty acid. Such high concentrations may perhaps even exist inside the cell and there exert a depressing effect.

THE SUPPLY OF PRECURSORS FOR THE EXTRAMITOCHONDRIAL SYNTHESIS OF FATTY ACIDS

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YRUVATE derived from the breakdown of carbohydrate, and fatty acids berated from fats, are oxidized to acetyl CoA in the mitochondria. Inder conditions of energy excess, much of the acetyl CoA so formed ıay be channeled into the synthesis of fatty acids (Green & Wakil, 960; Wakil, 1962). Distribution studies show that in liver and lac-ıting mammary gland of rat most of the synthesis of fatty acids up to almitate occurs extramitochondrially. How is the acetyl group of ıtramitochondrial acetyl CoA diverted from the mitochondria? Mole-ules of NADP remain in rat liver mitochondria for an average time of bout 85 minutes (Purvis & Lowenstein, 1961). Experiments similar ɔ those performed with NADP show that the rate of diffusion of CoA nd its acyl derivatives across the mitochondrial membrane *in vivo* is t least as slow as that observed with NADP. It can be calculated that ıch slow rates of diffusion of acetyl CoA are not rapid enough to ccount for the rates of fatty acid synthesis observed in the intact ssues. The experimental evidence for other pathways is considered elow.

(a) *Hydrolysis of acetyl CoA, and diffusion of acetate out of the mito-hondria.*—Acetyl CoA deacylase activities were measured by deter-ıining the amounts of acetate formed from acetyl CoA. Acetoacetate, -hydroxybutyrate, CoA, acetyl CoA and other acyl thiol esters do not ıterfere in the assay (Kornacker & Lowenstein, 1962, unpublished bservations). Acetyl CoA deacylase activities in mitochondria of liver ın account for between 10 and 50% of the maximum rate of fatty acid ynthesis depending on the nutritional state of the animal. The same nzyme in mitochondria of lactating mammary gland can account for nly 2% of the maximum calculated rate of fatty acid synthesis. Al-hough no measurements are available on the rate of diffusion of acetate ·om the mitochondria, this process would not be expected to be rate-miting with respect to fatty acid synthesis (compare below with itrate). The activities of the extramitochondrial (EM) acetate activa-ıng enzyme can account for 100% and 12% of the maximum rates of

fatty acid synthesis in liver and lactating mammary gland respectively. The enzyme activities in the pathway: intramitochondrial (IM) acetyl CoA → IM acetate → EM acetate → EM acetyl CoA, are therefore sufficient to account for 50% and 2% respectively of the maximum rates of fatty acid synthesis that occur in the intact tissues.

(b) *Conversion of acetyl CoA to citrate, and diffusion of citrate out of the mitochondria.*—The activity of the mitochondrial citrate condensing enzyme greatly exceeds the observed maximum rates of fatty acid synthesis. The rate of diffusion of citrate from mitochondria was measured under a variety of experimental conditions. In the presence of suitable substrates the rate of citrate extrusion from mitochondria is sufficient to account for the maximum rates of fatty acid synthesis observed in liver and lactating mammary gland of rat. Such citrate excretion occurs in the absence of mitochondrial swelling. Losses of endogenous citrate from mitochondria were previously reported by Schneider, Striebich & Hogeboom (1956). The activities of the extramitochondrial citrate cleavage enzyme can account for 100% and 40% respectively of the maximum rates of fatty acid synthesis in both tissues. The enzyme activities and diffusion rates in the pathway: IM acetyl CoA → IM citrate → EM citrate → EM acetyl CoA, can therefore account for these percentages of the maximum rates of fatty acid synthesis observed in the intact tissues.

(c) *Conversion of acetyl CoA to acetyl carnitine, and diffusion of this substance out of the mitochondria.*—Carnitine may have a carrier function in the oxidation of fatty acids by mitochondria (Bremer, 1962a, b). Moreover acetyl CoA can react with carnitine to yield acetyl carnitine (Friedman & Fraenkel, 1955). This acetyl transfer reaction was therefore assayed in suspensions of broken mitochondria. It occurs at a slower rate than the maximum rate of fatty acid synthesis. No measurements are available on the rate of diffusion of acetyl carnitine from the mitochondria, but the work of Bremer indicates that this would not be rate limiting with respect to fatty acid synthesis. The rate of incorporation of the acetyl group of acetyl carnitine into fatty acids was also measured. In the presence of high-speed supernatant preparations of lactating mammary gland this incorporation occurs, under optimum conditions, at about one-tenth of the rate of the incorporation of acetate and citrate. Competition experiments with acetate suggest that the acetyl group of acetyl carnitine may be converted to acetate before being incorporated into fatty acids. The enzyme activities in the pathway: IM acetyl CoA → IM acetyl carnitine → EM acetyl carnitine (→ EM acetate) → EM acetyl CoA, indicate that this is not the major pathway supplying acetyl groups for extramitochondrial fatty acid synthesis. This conclusion in no way detracts from the role for carnitine in fatty acid oxidation proposed by Bremer.

Citrate plays two main parts in fatty acid synthesis. It activates the acetyl CoA carboxylase reaction (Kallen & Lowenstein, 1962a;

artin & Vagelos, 1962; Waite & Wakil, 1963). At the same time, trate supplies acetyl CoA by the citrate cleavage reaction (Formica,)62; Kallen & Lowenstein, 1962b; Spencer & Lowenstein, 1962; ere & Bhaduri, 1962). The role of citrate as an activator thus leads its removal as a substrate. This constitutes a self-regulating mechasm for fatty acid synthesis, which in turn depends on the supply of trate (or its close biochemical relatives) from the mitochondria. The lative extents to which pathways (a) and (b) function in extramitoondrial fatty acid synthesis may vary with the foodstuff being conrted to acetyl CoA. In addition, pathways (a) and (b) may serve nctions other than to supply acetate and citrate for fatty acid synesis. For example, pathway (a) may supply acetate for acetoacetate or olesterol synthesis in the absence of fatty acid synthesis, and pathway) may supply isocitrate for the generation of extramitochondrial ADPH by the isocitrate dehydrogenase reaction.

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DISCUSSION

K. Stumpf: Have you considered the problems of the preparation of ver mitochondria? Hülsmann showed some time ago that the presence bile acids influences the permeability of the mitochondrial membranes enzymes.

M. Lowenstein: The problem that Hülsmann raises applies to the geon, not to the rat. The pigeon has no gall bladder and stores its bile parently in the liver, but the rat of course does not store bile in the ver. We have gone carefully into Hülsmann's criticisms. He says that nyone who finds fatty acid synthesis outside the mitochondria is erely liberating the enzymes from the mitochondria. The criteria we ave used is that the mitochondria should contain the full complement f pyridine nucleotides which they very easily lose. If they do contain

their full complement then they have not lost soluble enzymes. We have also measured the intramitochondrial malate dehydrogenase and the intramitochondrial isocitrate dehydrogenase activities. If these are not lost we assume that our mitochondrial preparations have not lost the fatty acid synthesizing enzymes. I should also say that the microsomes do not stimulate the mitochondrial system and that the microsomes alone give virtually no fatty acid synthesis.

H. Gutfreund: We are very interested in the various methods for determining the rate at which substrates and reagents get in and out of mitochondria, and we are particularly interested in the citrate experiments of Dr. Lowenstein. What I should like to know is whether his data allows him to calculate that proportion of citrate, which is synthesized in mitochondria by the condensing enzyme, which continues in the cycle as compared to the proportion which actually diffuses out into the extramitochondrial space. In mammary gland, this is likely to be fairly large because there is a lot of citrate excreted with milk. In liver, however, there is no such information available to us.

J. M. Lowenstein: I think we can calculate this ratio from our data but have not done so because we know the respiratory rate of the mitochondria. This rate must be almost entirely due to the operation of the citric acid cycle. From this we can calculate the rate of citrate formed versus the amount excreted.

D. C. Hardwick: It may be relevant to the last question to ask if you have any hypothesis on the fate of the oxaloacetate. A lot of it is formed in the mammary gland. Does it get back in the mitochondria?

J. M. Lowenstein: One can postulate various carrier mechanisms to get the acetyl group across the mitochondrial membranes and as I said we tested this with acetyl carnitine and carnitine is probably not the carrier. We look upon oxaloacetate as the acetyl carrier. Now of course it may be reduced to malate if it goes back in the mitochondria, the DPNH/DPN ratio suggests that this happens. Then in the mitochondria we would have to have reoxidation and reconversion in citrate. Nevertheless we look on it as a carrier.

M. N. Berry: In experiments in which liver homogenate is incubated with pyruvate it is possible to decrease the rate of oxaloacetate synthesis. Under these conditions citrate formation is impaired and ketone bodies accumulate. However, isotopic data indicate that the oxidation of acetyl CoA through the tricarboxylic acid cycle may proceed at normal rates under these conditions.

If the formation of citrate represents the first step in fatty acid synthesis, this might be impaired by oxaloacetate deficiency. Transfer of acetyl CoA to the cytoplasmic compartment of the cell would be impeded

and instead acetyl CoA would accumulate within the mitochondria and be converted to ketone bodies by an intramitochondrial enzyme system. The severity of the ketosis would reflect the degree of impairment of liver fatty acid synthesis.

J. M. Lowenstein: Yes, I have one comment to make on that. We have shown that citrate is a very good precursor for the extramitochondrial synthesis of acetoacetate so we are essentially in agreement on this.

S. Smith: How does the ratio of microsomes to cell sap (supernatant preparation) at optimum microsomal stimulation compare with the ratio in the cell?

J. M. Lowenstein: There is slightly more "microsomal material" under the optimum conditions than found in the whole cell.

FACTORS INFLUENCING FORMATION AND EXCRETION OF BILE ACIDS

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The two main primary bile acids formed from cholesterol in most mammalian species are cholic (IV) and chenodeoxycholic (VII) acids. In the species that have been examined, man, rat, mouse and rabbit, these acids are not metabolically interconvertible. The formation of chenodeoxycholic acid from cholesterol in the rabbit has not been conclusively demonstrated but several lines of evidence strongly indicate that this reaction occurs to some extent in this species.

MECHANISMS OF BILE ACID FORMATION

The mechanism of formation of bile acids has been studied *in vivo* as well as *in vitro*, with, in most cases, the rat as the experimental animal. The sequence and mechanisms of the reactions leading from cholesterol to cholic and chenodeoxycholic acids are not known in detail. A number of investigations on the metabolism of hypothetical intermediates in the bile fistula rat have yielded important information. On the basis of these results and of others obtained *in vitro* using cholesterol and other C_{27}-neutral steroids it is possible to advance a hypothesis on the sequence of reactions in bile acid formation. Figure 1 shows the probable early steps in the formation of cholic and chenodeoxycholic acids. The mechanism of the degradation of the cholesterol side-chain has not been completely established. The conversion of 5β-cholestane-3α,7α,12α-triol (VIII) to cholic acid has been well studied and has been shown to entail an ω-oxidation followed by a "β"-oxidation yielding cholyl-CoA (XII) and propionyl-CoA (cf. Fig. 2). Results have been obtained which indicate that the oxidation of the cholesterol side-chain proceeds by the same mechanisms. The existence of another pathway for the degradation of the cholesterol side-chain has been demonstrated by Whitehouse, Staple & Gurin (1961) who found that mitochondrial preparations catalyzed the cleavage of the cholesterol side-chain, with the formation of acetone originating from carbons – 25, 26 and 27 of cholesterol. The quantitative importance of this reaction in bile acid biogenesis is not known. The mechanisms of bile acid formation have been

the subject of a recent review which contains pertinent references (Danielsson, 1963).

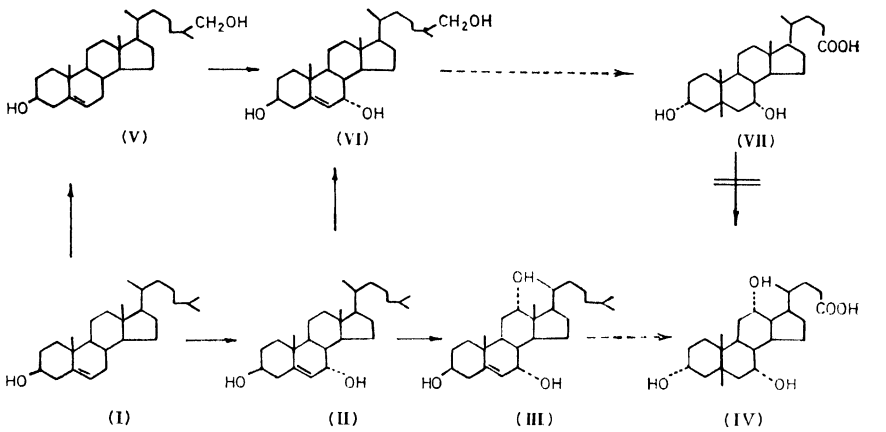


Fig. 1. Proposed scheme for the conversion of cholesterol (I) to cholic (IV) and chenodeoxycholic (VII) acids.
7α-Hydroxy-cholesterol (II); cholest-5-ene-3β, 7α, 12α-triol (III); 26-hydroxycholesterol (V); cholest-5-ene-3β, 7α, 26-triol (VI).

METABOLISM AND EXCRETION OF BILE ACIDS

In the bile of mammals the bile acids are present as conjugates of glycine and taurine. The proportion of glycine to taurine conjugates varies with species. In the rat the bile acids are conjugated predominantly with taurine, in rabbit with glycine and in man the ratio of glycine to taurine conjugated bile acids is about 3. During the entero-

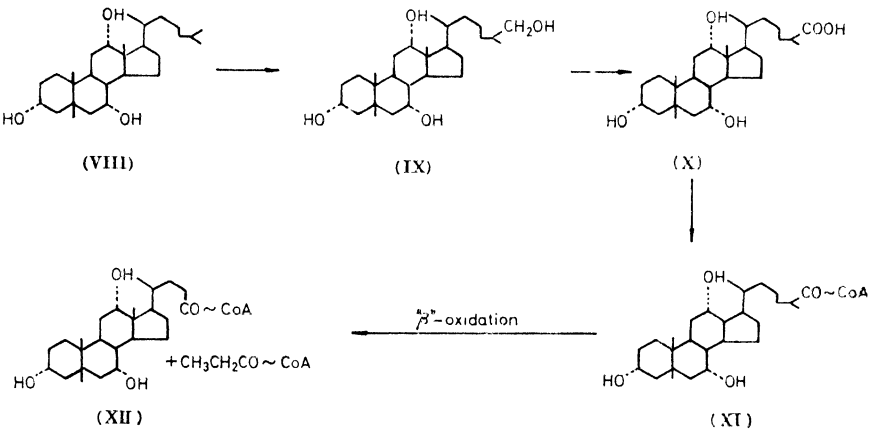


Fig. 2. Proposed scheme for the mechanism of the conversion of 5β-cholestane-3α, 7α, 12α-triol (VIII) to cholyl-CoA (XII).
5β-Cholestane-3α, 7α, 12α, 26-tetrol (IX); 3α, 7α, 12α-trihydroxy-5β-cholestanoic acid (X).

hepatic circulation of bile the primary bile acids are modified by intestinal microorganisms in the caecum and colon. The initial reactions consist of removal of the 7α-hydroxyl group from the primary bile acids and of hydrolysis of the conjugates yielding the free bile acids. The acids formed, deoxycholic (XIII) and lithocholic (XIV) can be reabsorbed, but to markedly different extents. Deoxycholic acid is well absorbed. It is the main bile acid in gall bladder bile of the rabbit and constitutes about 20% of the bile acids in human bile. In the rat absorbed deoxycholic acid is reconverted to cholic acid by a 7α-hydroxylase in the liver, and is practically absent from rat bile. In contrast to deoxycholic acid, lithocholic acid is poorly reabsorbed under normal conditions, i.e. under the conditions of its formation *in vivo*, and is present in bile only in traces. Through the work of Gustafsson & Norman (1962), Norman & Shorb (1962) and Norman (to be published) the reasons for this difference in extent of reabsorption of deoxycholic and lithocholic acids have become apparent. By subjecting intestinal contents of rats and suspensions of human faeces to differential centrifugation it has been shown that lithocholic acid remains in the sediment while a considerable part of the deoxycholic acid is present in the clear 25,000 × g supernatant fluid. Lithocholic acid can be extracted from the sediment only with solvents such as acetone, indicating that it is firmly adsorbed to the water-insoluble residue or bound intracellularly in the bacteria.

Similar mechanisms are probably responsible for the difference in composition of the biliary bile acids and the faecal bile acids. In contrast to the simple and uniform composition of the bile acid fraction of bile, the faecal bile acid fraction represents a rather complex mixture of microbial metabolites of the biliary bile acids. The composition of the faecal bile acid fraction is dependent on the composition of the intestinal flora and is influenced by factors which cause changes in the intestinal flora, e.g. antibiotics and diet. The results of work on methods suitable for quantitation of the bile acids excreted in rabbit and human faeces will be published by Eneroth, Hellström & Sjövall. The main faecal bile acids have been isolated and identified (Danielsson, Eneroth, Hellström & Sjövall, 1962; Danielsson, Eneroth, Hellström, Lindstedt & Sjövall, 1963; Danielsson, Kallner & Sjövall, 1963). Hydrolyzed ethanol or chloroform/methanol extracts of human faeces have been subjected to chromatography on silicic acid columns yielding a fatty acid, a sterol and a bile acid fraction. The methylated bile acid fraction was further purified by chromatography on columns of aluminum oxide. Six main bile acids were isolated: 3β-hydroxy-5β-cholanic acid (XIX), lithocholic acid (XIV), 3β-hydroxy-12-keto-5β-cholanic acid (XVII), 3α-hydroxy-12-keto-5β-cholanic acid (XVI), 3β,12α-dihydroxy-5β-cholanic acid (XV), and deoxycholic acid (XIII) (cf. Fig. 3). In addition, 3-keto-5β-cholanic acid (XVIII) and chenodeoxycholic acid (VII) were also present as judged from chromatographic data. Small amounts of cholic acid (IV)

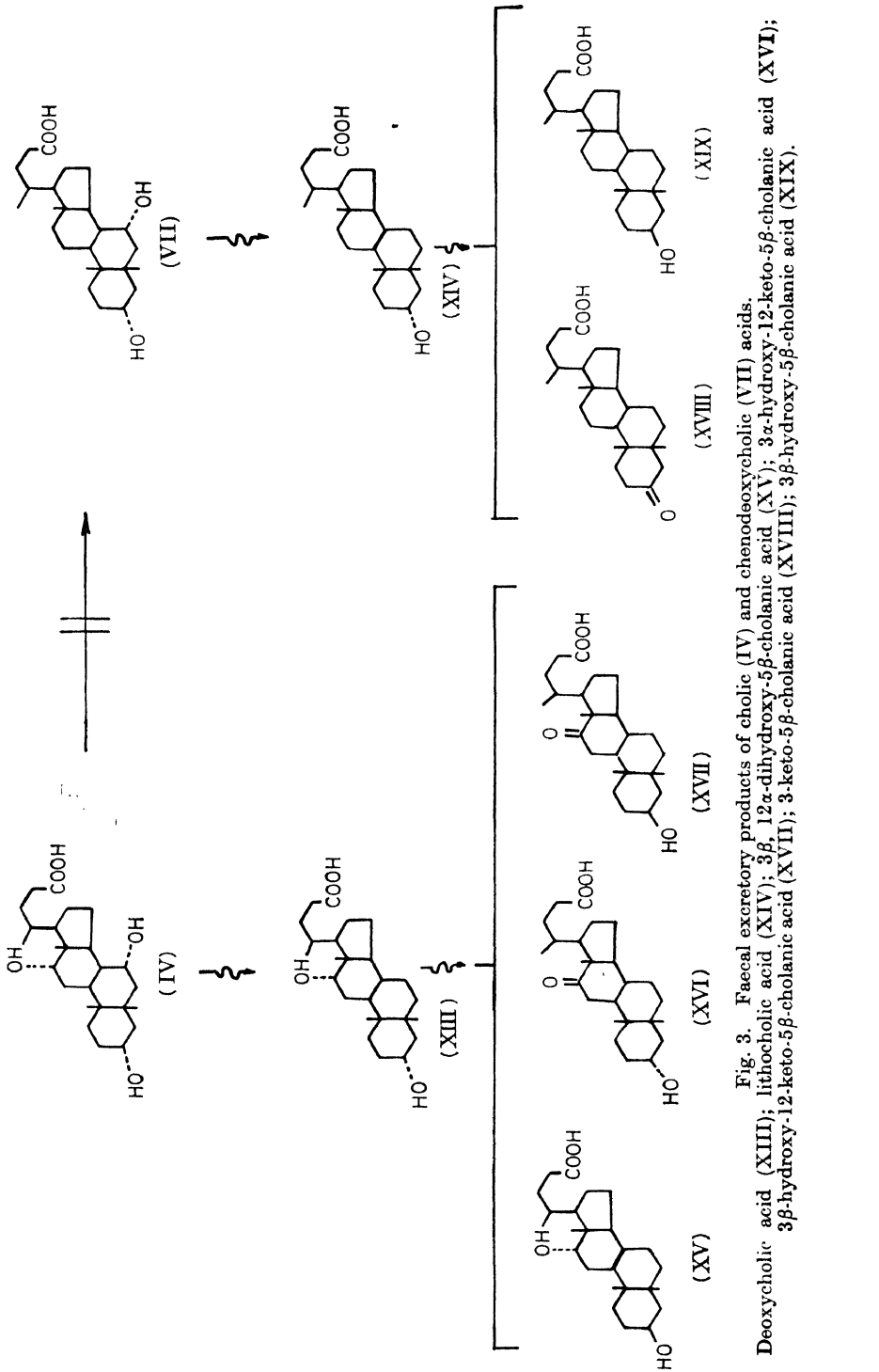


Fig. 3. Faecal excretory products of cholic (IV) and chenodeoxycholic (VII) acids.
Deoxycholic acid (XIII); lithocholic acid (XIV); 3β, 12α-dihydroxy-5β-cholanic acid (XV); 3α-hydroxy-12-keto-5β-cholanic acid (XVI); 3β-hydroxy-12-keto-5β-cholanic acid (XVII); 3-keto-5β-cholanic acid (XVIII); 3β-hydroxy-5β-cholanic acid (XIX).

can sometimes be detected in extracts of human faeces and it is probable that other, as yet unknown, bile acids are present in small amounts. It should be pointed out that in extracts of faeces that have not been subjected to saponification, some bile acids can be present as less polar derivatives (Norman, to be published). This fact has not been taken into account in some of the published methods for quantitation of faecal bile acids. The composition of the bile acid fraction of rabbit faeces is similar to that of human faeces and the same six main bile acids (cf. Fig. 3) have been identified (Danielsson *et al.* 1962; Danielsson, Kallner & Sjövall, 1963). In addition, 3α,12α-dihydroxy-5α-cholanic acid has been identified as a minor component. This acid, which is formed from deoxycholic acid by microbial enzymes, is reabsorbed and is present in bile in small amounts. It is apparently identical with the β-lagodeoxycholic acid isolated from rabbit bile by Kishi in 1936.

By isotopic techniques Norman & Sjövall (1958, 1960) have demonstrated that in the rat lithocholic, deoxycholic, 3α-hydroxy-12-keto-5β-cholanic and 3α,12α-dihydroxy-7-keto-5β-cholanic acids are major faecal bile acids. However, chromatographic data indicate the presence of several other bile acids in rat faeces.

TURNOVER OF BILE ACIDS

According to current concepts of cholesterol metabolism the steroid ring system cannot be degraded either by enzyme systems in the tissues or by those of normal intestinal microorganisms. The main end-products of cholesterol metabolism, bile acids and neutral C_{27}-steroids, are eliminated mainly with the faeces. Small amounts of bile acids are excreted in urine. The extent of urinary excretion of bile acids as compared with that of faecal excretion varies with species and is apparently correlated to the level of bile acids in blood (Hellström & Sjövall, 1962). Urinary excretion of bile acids is negligible in the rat. In the rabbit it represents about 10% and in man about 5% of total bile acid excretion under normal conditions. Provided that no degradation of the bile acids occurs in the intestinal tract, the amount of bile acids excreted in faeces will thus practically equal the synthesis of bile acids. In view of the complexity of the bile acid mixture excreted in faeces, determination of faecal bile acid excretion by non-isotopic methods poses a difficult analytical problem. Bile acid turnover has been measured by isotope techniques in several species and under different hormonal and dietary conditions. However, as a complement to these data it would be of interest to obtain information on the influence of different factors on the pattern of bile acid excretion in faeces. Work on methods suitable for analysis of the different faecal bile acids is in progress.

In rats fed a chow-diet the half-life of bile acids is 2–3 days as measured by following faecal excretion of isotope. The daily production is about 5 mg as calculated from the half-life and from the size of the bile acid pool. In rabbits fed a chow-diet the half-life of deoxycholic

acid is about 7 days and the daily production about 80 mg (Hellström & Sjövall, 1962). The half-life was determined by following the decline in specific activity of the biliary glycodeoxycholic acid after administration of labelled deoxycholic acid, and the size of the glycodeoxycholic acid pool by calculating the dilution of the administered isotope. Samples of bile were collected by duodenal intubation after intravenous administration of cholecystokinine. This method of determining bile acid production was originally developed by Lindstedt (1957) for studies of bile acid production in man. In connection with their study of the turnover of deoxycholic acid in rabbits, Hellström & Sjövall (1962) also measured the urinary and faecal excretion of isotope. The daily excretion of bile acids was calculated by dividing the total radioactivity excreted during a certain period of time with the mean specific activity of the biliary glycodeoxycholic acid during the same period of time. The daily excretion of bile acids determined in this manner amounted to 80–90% of the calculated daily formation of bile acids. The reasons for this discrepancy are not known and work is in progress to examine further the metabolism of bile acids in the intestinal tract. In man the turnover of cholic acid has been determined in a number of subjects by Lindstedt (1957, 1962). In healthy subjects on an *ad libitum* diet, the half-life of cholic acid is about 3 days and the daily production about 350 mg. The total daily production of bile acids has been calculated to be 500–700 mg (Lindstedt, 1957, 1962; Danielsson *et al.*, 1963). Rosenfeld & Hellman (1962) have reported that the total daily excretion of bile acids in faeces of man as determined by isotope techniques amounts to about 300 mg. At present, investigations are being carried out in this laboratory to determine simultaneously the production and excretion of bile acids in man.

REGULATION OF BILE ACID FORMATION

Bergström & Danielsson (1958) have studied the effect of duodenal infusion of bile salts on the synthesis of bile acids in bile fistula rats and have obtained results indicating that bile acid formation is homeostatically regulated by the concentration of bile acids in the portal blood. Studying the effect of bile acid feeding on cholesterol synthesis and catabolism in rats and mice, Beher, Baker, Anthony & Beher (1961) have obtained evidence for the existence of a double feed-back mechanism controlling bile acid formation. Feeding of cholic acid inhibits conversion of hepatic cholesterol to bile acids leading to an increase in hepatic cholesterol level which in turn inhibits hepatic cholesterol synthesis. Portman (1962) has obtained results indicating that in order to influence cholesterol synthesis a bile acid must meet certain structural requirements. Thus, triformoxycholic acid was found to have no effect on cholesterol synthesis while cholic acid inhibited cholesterol synthesis markedly. At present, studies are in progress on bile acid synthesis in bile fistula rats that are continually infused with different bile acids via the portal vein.

INFLUENCE OF HORMONES ON BILE ACID METABOLISM

The influence of thyroid activity on bile acid formation has been studied in rat and in man. Eriksson (1957) found that the total excretion of bile acids in bile fistula rats was the same in hyperthyroid as in euthyroid rats, while the hypothyroid state was associated with a marked decrease in bile acid excretion. In the hyperthyroid state the ratio of cholic to chenodeoxycholic acid was almost the reverse of that found in euthyroid rats, indicating that thyroid hormones exert a direct effect on bile acid formation. This contention is further strengthened by recent work of Strand (1962) who has shown that the same reversal of the ratio of cholic to chenodeoxycholic acid is induced by D-triiodothyronine in non-calorigenic doses. Preliminary data indicate that 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol in rat liver homogenates is stimulated by prior administration of thyroid hormones. Strand (1963) has recently concluded a study on the effect of thyroid hormones and analogues on bile acid production in intact rats. As shown in Table 1,

Table 1. *Turnover of bile acids in rats treated with* L-*triiodothyronine,* D-*triiodothyronine and propylthiouracil*

Group	Half-life (days)	Pool size of cholic acid (mg)	Pool size of chenodeoxycholic acid (mg)	Daily production of cholic acid (mg)	Daily production of chenodeoxycholic acid (mg)	Total daily production (mg)
Normal (mean of five animals)	2·3	12·6	3·2	4·0	1·0	5·0
Rats treated with L-triiodothyronine (40 μg/kg/day) (mean of four animals)	2·1	16·6	7·0	5·6	2·3	8·0
Rats treated with L-triiodothyronine (200 μg/kg/day) (mean of six animals)	2·1	13·5	10·2	4·6	3·4	7·9
Rats treated with D-triiodothyronine (40 μg/kg/day) (mean of four animals)	1·7	13·5	6·7	5·5	2·9	8·4
Rats treated with propylthiouracil (mean of three animals)	2·4	12·6	3·7	3·6	1·0	4·6

daily production of bile acids is higher in rats treated with L- or D-triiodothyronine in non-calorigenic doses than it is in euthyroid rats, while treatment with propylthiouracil does not influence bile acid production.

In man, hypothyroidism is associated with an increase in the ratio of glycine to taurine conjugated bile acids in bile (Hellström & Sjövall, 1961). The ratio of cholic to chenodeoxycholic acid is not influenced by thyroid activity to the same extent as it is in rats. A slight decrease in

the normal ratio of cholic to chenodeoxycholic acid has been observed in hyperthyroid patients (Hellström & Lindstedt, to be published). The daily production of cholic acid has been determined in a number of hyper- and hypothyroid patients by Hellström & Lindstedt (to be published). Cholic acid production was lower in the hypothyroid than in the hyperthyroid patients and after treatment cholic acid production increased markedly in most of the hypothyroid patients (cf. Table 2).

Table 2. *Influence of thyroid activity on turnover of cholic acid in man.*

Group	Half-life (days)	Pool size (g)	Daily production (g)
Hypothyroid patients (mean values for 9 patients)	3·6	1·06	0·22
Hyperthyroid patients (mean values for 7 patients)	1·9	0·91	0·34

Few data are available on the effects of other hormones on bile acid metabolism. Hellström & Strand (1963) have found that in bile from adrenalectomized rats the ratio of taurine to glycine conjugated bile acids is about 1 as compared with about 9 in normal rats. Cortisone treatment of adrenalectomized rats restores the conjugation pattern to normal. Kritchevsky, Staple, Rabinowitz & Whitehouse (1961) have observed that mitochondria from female rats are considerably more active than those from male rats with respect to capacity to oxidize 26-[^{14}C]-cholesterol to labelled carbon dioxide.

INFLUENCE OF INTESTINAL MICROORGANISMS ON BILE ACID METABOLISM

The effects of intestinal microorganisms on metabolism and excretion of bile acids were discussed above from the qualitative point of view. Quantitatively, the turnover of bile acids is influenced markedly by the intestinal flora. In rats treated with antibiotics or reared germ-free the half-life of cholic acid is 3-5 times longer than in conventional rats and the daily production of cholic acid in germ-free rats is about half that in conventional rats (Lindstedt & Norman, 1956; Gustafsson, Bergström, Lindstedt & Norman, 1957; Gustafsson, Norman & Sjövall, 1960). It is possible that in the absence of intestinal microorganisms more bile acids are available per unit of time for reabsorption leading to a decrease in bile acid synthesis in the liver.

INFLUENCE OF DIET ON BILE ACID METABOLISM

The effects of different diets on bile acid turnover have been studied in rat, rabbit and man. In rats and rabbits, a change from a chow-diet to a semi-synthetic diet is associated with a marked increase in half-life time and a decrease in daily production of bile acids (Portman, 1960;

Hellström, Sjövall & Wigand, 1962). A similar effect is observed in man when a change is made from an *ad libitum* diet to a formula diet (Lindstedt & Steinberg, to be published). Addition of indigestible residue to the diet causes a marked increase in cholic acid production in rats (Portman, 1960). Whether these effects depend on a change in intestinal flora or in the rate of passage of contents through the intestinal tract—or both—is not known. Both factors might influence the amount of bile acids available for reabsorption. No detailed data have been published concerning the effects of different diets on the composition of bile acids in intestinal contents of faeces.

In recent years a number of reports have been published dealing with the mechanisms by which saturated and unsaturated fats influence serum cholesterol levels. Much of the work has been concerned with the influence of feeding saturated and unsaturated fat on cholesterol catabolism and excretion and specifically on excretion of bile acids and neutral sterols in faeces. Wilson & Siperstein (1959) found an increase in faecal excretion of nondigitonin-precipitable sterols in rats fed corn oil as compared with rats fed lard or a fat-free diet. Ferguson, Coniglio & Blood (1961) found no differences in faecal excretion of bile acids or sterols between rats fed saturated and those fed unsaturated fat nor did they observe any differences in the distribution of cholesterol in the body. Lambiotte (1962) has obtained similar results. Gerson, Shorland & Adams (1961) have found that in rats addition of corn oil to a low-fat diet leads to an increase in cholesterol concentrations of tissues concomitant with a drop in serum cholesterol level.

In rabbits, the turnover of deoxycholic acid has been found to be the same whether corn oil or hydrogenated cocoanut oil is included in a semi-synthetic diet (Hellström, Sjövall & Wigand, 1962).

Hellman & Rosenfeld (1959) have obtained results indicating that in man feeding of unsaturated fat is associated with an increase in faecal excretion of neutral sterols and no change in excretion of bile acids. Goldsmith, Hamilton & Miller (1960) found an increase in faecal excretion of both bile acids and neutral sterols on changing from saturated to unsaturated fat. Lindstedt & Steinberg (to be published) have found that in subjects fed a formula diet containing corn oil or cocoanut oil the daily production of cholic acid is greater on the corn oil diet than on the cocoanut oil diet.

It seems apparent that further work is needed to clarify the effects of different fats on metabolism and excretion of cholesterol. At present, synthesis and excretion of bile acids is being studied in a group of healthy subjects fed standardized solid diets with 40% of the calories as fat, either in the form of butter fat or corn oil.

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DISCUSSION

N. B. Myant: Do you think there are two pathways for the conversion of cholesterol to bile acids: one shown in Fig. 1, in which the side-chain is oxidized before hydroxylation of the ring system and saturation of the 5 : 6 double bond, and the other shown in Fig. 2? Would you not agree that there is a good deal of evidence that trihydroxycoprostane, which comes into Fig. 2 pathway, is a normal intermediate. If not, then what is the relevance of the *in vitro* experiment, which you quote later, in which trihydroxycoprostane is used as a precursor for studying the effect of thyroid hormone on mitochondrial formation of bile acids from cholesterol?

S. Bergström: I think both pathways are possible, and I think that normally the one through trihydroxycoprostane is the predominating

one. The mechanism of oxidation of sterols is a very unspecific one. You can put in any type of steroid and get oxidation, depending more or less on the structure, whereas cholesterol is handled predominantly via the trihydroxycoprostane route. But we are not yet quite certain about the quantitative relation between the two pathways in the oxidation of cholesterol.

N. B. Myant: Do you think that trihydroxycoprostane which you show in Fig. 2, does come into the Fig. 1 scheme as well? If so, do you think it is a normal intermediate in bile acid formation *in vivo*?

S. Bergström: Yes. Not necessarily 100%, but the predominating one.

W. M. Whitehouse: You mentioned that you have been studying the effect of bile acids other than deoxycholate in controlling the feed-back mechanism. Have you discerned any particular differences in the efficacy of different bile salts?

S. Bergström: We do not have anything to report so far. We happen to have used this intraportal continuous infusion. With this you really run into trouble, because when the bile acids are injected directly they cause haemolysis. Whereas if they are absorbed via the intestine apparently they are taken up by albumin before they get in contact with erythrocytes. I hope we shall report a little later on this point.

THE ROLE OF REDUCED COENZYMES AND OXYGEN IN THE CONTROL OF FATTY ACID SYNTHESIS IN ADIPOSE TISSUE

By J. P. FLATT* and E. G. BALL

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Epididymal adipose tissue from three rats was randomly distributed among three pairs of flasks and incubated at 37° for two hours in Krebs-Henseleit bicarbonate medium. One flask of each pair contained 10mM ^{14}C-glucose labeled uniformly and the other the same amount of glucose labeled specifically in the C-6 position. One pair of flasks served as controls, another contained insulin (1000 μunits/ml) while the third contained insulin and epinephrine (0·2 μg/ml). Net gas exchange, total CO_2 production and oxygen consumption were determined manometrically, and glucose uptake, lactic acid and glycerol production measured. Radioactive carbon incorporation into the defatted tissue residue (=glycogen), CO_2, fatty acids, and glyceride-glycerol was also determined. The assumptions are made that ^{14}C from the C-6 position of glucose is converted to $^{14}CO_2$ only in the citric acid cycle and that fatty acid is the only precursor other than glucose which contributes significantly to the CO_2 pool. A set of equations may then be derived which, with the data in hand, permit the calculation of the amount of CO_2 produced respectively in (1) the pentose cycle (2) the decarboxylation of pyruvate and (3) the citric acid cycle. Of critical importance for these calculations is the ratio of ^{14}C incorporated into fatty acids from the two labeled glucose precursors. An accurate graphic interpolation of this ratio was made possible by the finding that incorporation of ^{14}C into fatty acids is proportional to the net gas exchange (corrected for lactic acid release). The information obtained on the amount of CO_2 produced in the three loci permits in turn the calculation of the μatoms of carbon flowing through the various metabolic pathways under study in adipose tissue. A check of the validity of the calculation is afforded by the fact that the flow of glucose-carbon into the acetyl-CoA pool, as determined by the amount of CO_2 calculated to have been produced by decarboxylation of pyruvate, agrees within 2 to 7% of that leaving

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the pool measured as the sum of glucose-carbon incorporated into fatty acids and metabolised to CO_2 in the citric acid cycle. An estimation of the quantity of reduced coenzymes both produced and utilized in the various pathways is also possible.

The addition of insulin caused the following increases over the control values: glucose uptake, 300%; CO_2 production, 200%; O_2 consumption, 80%; fatty acid synthesis, 560%. In the control tissue 50% of the total CO_2 is produced in the citric acid cycle while in the presence of insulin only 7% of the total is so produced. Hence, despite the increase in oxygen consumption which accompanies the stimulation of fatty acid synthesis, there is a marked diminution in activity of the citric acid cycle. In the presence of insulin the reduced coenzymes produced, expressed as percentage of the total, may be assigned to four chief loci as follows: pentose cycle, 30%; glyceraldehyde phosphate dehydrogenase, 33%; pyruvic acid oxidase, 30%; citric acid cycle, 7%. Utilization is as follows: lactate and glycerophosphate production, 5%; fatty acid synthesis, 50%; the remaining 45% of the reduced coenzymes being reoxidized by oxygen.

The NADPH formed in the pentose cycle is sufficient to supply only about half of the reduced coenzymes needed for fatty acid synthesis (60% with insulin; 50% with insulin + epinephrine). The other half of the reducing equivalents needed for this process must therefore be furnished by reduced coenzymes produced during the conversion of triose phosphate to acetyl-CoA. The results suggest, though do not prove, that in intact adipose tissue only one step in the reduction of acetyl-CoA to fatty acid specifically requires NADPH. Moreover the amounts of reduced coenzymes generated in the conversion of triose phosphate to acetyl-CoA are far in excess of those needed to complete the reductive formation of fatty acids. Thus, the process of converting glucose into fat leads to the obligatory formation of a surplus of reduced coenzymes. These must be reoxidized if fatty acid synthesis is to proceed and the partial shutdown of the citric acid cycle may thus be a compensatory mechanism to permit this reoxidation to proceed.

In the presence of epinephrine in addition to insulin more lactate and glycerophosphate are formed. The production of the latter is increased by 70% while the glycerol released from triglycerides increases to about the same extent. This finding reflects the well-known ability of epinephrine to stimulate lipolysis in adipose tissue. Since glucose and insulin are both present the free fatty acids released are reesterified with the glycerophosphate formed. There is a concomitant increase in O_2 consumption (13%) accompanied by an increase in the CO_2 released in the citric acid cycle which is of the magnitude to account for the additional O_2 uptake. Of special interest is the fact that fatty acid synthesis is increased by 11% (0·5 μmoles of acetyl-CoA/100 mg wet weight/2 hours). Whether this is a reflection of the increased use of reduced coenzymes for lactate and glycerophosphate formation is not known.

However, one might expect that any procedure which enhances the oxidation of the reduced coenzymes which are produced in excess of those needed for fatty acid synthesis would favor this synthesis.

In conclusion it appears that in intact adipose tissue one controlling factor in the synthesis of fatty acids from glucose is the tissue's ability to oxidize the surplus reduced coenzymes which are generated in this process.

DISCUSSION

S. J. Folley: Do you find that the net gas exchange is proportional to the incorporation of glucose carbon into fatty acid? If so, I take it that the RQ was greater than 1, as it is in mammary gland. We have never found this to be exactly so, but years ago we used to talk of extra CO_2 and I always thought that this gave a measure of the lipogenesis.

J. P. Flatt: Yes. We measured the CO_2 production and the oxygen consumption and the RQ in the presence of insulin is almost exactly two.

G. B. Phillips: In this connection I would like to mention the work of my colleagues in Columbia who found that in the epidermal fat pad of the rat, in the absence of oxygen, there is a marked selective inhibition of the synthesis of palmitic, oleic and linoleic acids. There is also a complete inhibition of the desaturation of stearic to oleic acid. Almost exactly the same situation was found in the epidermal fat pad of the rat made diabetic with alloxan. These abnormalities cannot be rectified by the addition of reduced pyridine nucleotides. I wonder therefore whether the increase in oxygen consumption following insulin administration might be related, at least to some extent, to a stimulation of this desaturation reaction, which apparently requires reduced coenzyme.

J. P. Flatt: The increase in oxygen consumption is mainly due to the disposal of a great excess of reduced coenzymes. We have no further details of the desaturation mechanism, but it is quantitatively accounted for by the excess production of reduced coenzymes in the conversion of glucose to fatty acids.

HORMONES AND CHOLESTEROL METABOLISM

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INTRODUCTION

All nucleated mammalian cells appear to have the ability to synthesize cholesterol. The complex synthetic pathway (Popjak & Cornforth, 1960) appears to be controlled by various mechanisms only one of which is hormonal.

To investigate the endocrine control of metabolic processes it is frequently necessary to delete specific endocrine organs. However, removal of any one endocrine organ results in alterations to other endocrine systems because the endocrine glands function essentially in an integrated manner and this complicates the interpretation of most experiments of this type.

There is evidence in mammals that the liver is an important organ in the biosynthesis of cholesterol and the hepatic cholesterol equilibrates rapidly with the plasma cholesterol. The plasma cholesterol occurs as a heterogeneous series of lipoproteins and in each lipoprotein the cholesterol exists as nonesterified and esterified cholesterol. Cholesterol is degraded to neutral sterols, bile acids, vitamin D and a variety of steroid hormones. Quantitatively the excretion of neutral steroids and bile acids far exceeds that of any of the other degradation products.

The bile acids undergo an entero-hepatic circulation (Bergstrom, Danielsson & Samuelsson, 1960) and are eventually modified further by the intestinal flora found in the large intestine prior to faecal excretion. A number of factors, dietary, hormonal, pharmacological and environmental have been studied which affect cholesterol synthesis but we have little information on the factors which dictate the partitioning of cholesterol between various tissues and body fluids.

This review will be concerned with a restricted series of hormones, thyroactive and oestrogenic, and their effect on a few facets of cholesterol metabolism in two species—the rat and man. In order to keep endocrine effects in perspective these studies have been contrasted with the effect of fasting.

The animals used, the diets fed, etc. in unpublished work cited in this paper are similar to those described previously (Boyd, 1962).

Rates of sterol synthesis *in vivo* have been studied using [1-^{14}C]-acetate and [2-^{14}C]-mevalonate as the cholesterol precursors which were usually administered intraperitoneally. The cholesterol in tissues and body fluids was isolated and counted by liquid scintillation techniques as described previously (Boyd, 1962).

In the studies involving labelling of cholesterol using acetate, observations were also made on the rate of evolution of $^{14}CO_2$ expired by the experimental animals. The animals were placed in an all-glass metabolism unit immediately after receiving the acetate and air was drawn through the metabolism chamber. The respiratory $^{14}CO_2$ was collected in aqueous potassium hydroxide absorption vessels. The contents of the vessels were changed hourly and the $^{14}CO_2$ collection was usually maintained for 24 hr. Aliquots of the potassium hydroxide solution were taken for C^{14} assay as barium carbonate, at infinite thickness, using a Geiger Müller end window counting technique. A typical result obtained in an experiment with normal male rats is shown in Fig. 1. The results

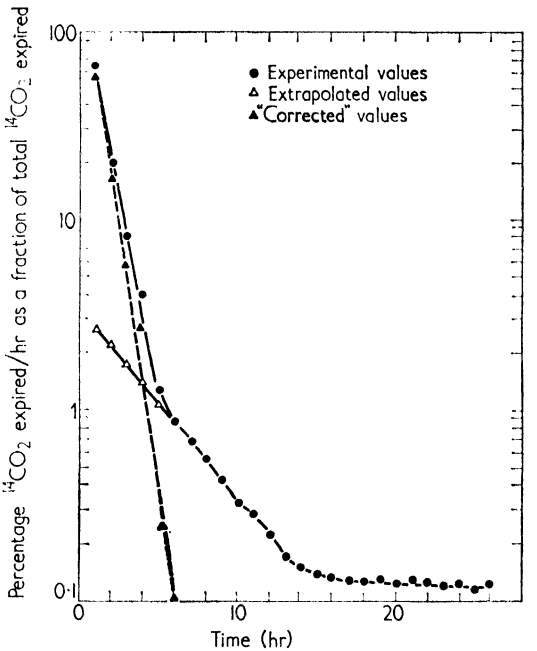


Fig. 1. The $^{14}CO_2$ expired by male rats injected intraperitoneally with [1-^{14}C]-acetate at zero time. ● experimental values; △ extrapolated values; ▲ experimental points corrected for the extrapolated values.

are expressed in $^{14}CO_2$ excreted per hr. as a percentage of the total $^{14}CO_2$ excreted over the 24 hr. period. This graph can be resolved into two exponential components by extrapolation of the curve back to zero time, followed by "correction" of the initial phase of the graph as shown in Fig. 1.

In attempting to study cholesterol degradation, use was made of the fact that most of the "bile acid pool" in the rat is to be found in the intestine. Using the methods which we devised for the determination of bile acids in plasma (Reid & Boyd, 1958) it is possible to isolate the bile acids according to their polarity by the method of Matschiner, Mahowald, Elliott, Doisy, Hsia & Doisy (1957). When animals have been injected with [2-^{14}C]-mevalonate prior to killing it is possible to determine the specific activity of the liver cholesterol and the intestinal bile acids and so obtain a measure of cholesterol degradation to bile acids over a specific interval of time. In most of the studies described here the animals were allowed to survive 24 hr. after the administration of mevalonate. A typical result obtained in the separation of [^{14}C] bile acids is shown in Fig. 2.

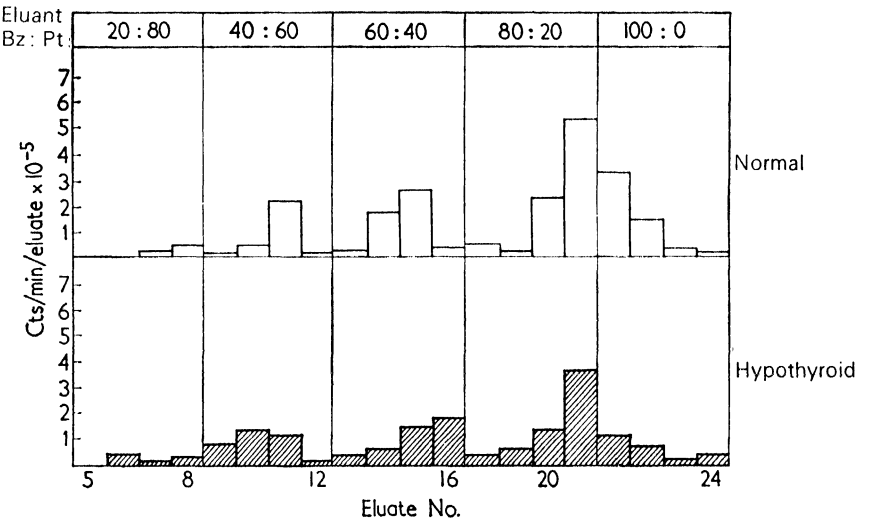


Fig. 2. The chromatographic separation of the small intestinal bile acids using the method of Matschiner *et al.* (1957) which employs benzene: petrol ether :: 70% acetic acid. The open histograms are from normal animals and the hatched histograms are from hypothyroid animals which were injected previously with [2-^{14}C]-mevalonate.

Studies have been made of the concentration of cholesterol in tissues and body fluids in various endocrine states. The distribution of cholesterol between various esters has been investigated by various authors (Riley & Nunn, 1960; Schrade, Biegler & Bohle, 1961) using silicic acid chromatographic isolation of the esters (Hirsch & Ahrens, 1958) and the determination of the fatty acid composition of the esters using gas-liquid chromatography (James, 1960).

In the studies involving thyroid hormones it has been necessary to determine the activity of the hormone using oxygen consumption rate and heart rate as parameters of metabolic stimulation. The methods involved in these studies have been described before (Boyd & Oliver, 1960).

In the case of studies on the phagocytic activity of the reticulo-endothelial system (RES) the particulate carbon clearance method of Biozzi, Benacerraf & Halpern (1953) has been used as discussed previously (Boyd, 1962).

OESTROGENS

Effects of ovariectomy

In the female human and rat ovariectomy produces an increase in the plasma cholesterol concentration of about 15 to 30% of the preoperative value (Oliver & Boyd, 1959; Boyd, 1961).

The rise in plasma cholesterol in both species after oestrogen withdrawal is largely due to an increase in the β-lipoprotein and this seems to be true both in the rat and in the human.

The plasma ester cholesterol composition is heterogeneous and the cholesterol ester pattern also appears to change after ovariectomy. The results of a study on the plasma cholesterol ester composition before and after ovariectomy is shown in Table 1. For simplicity only some of the fatty acids have been shown in this table; it will be noted that there is a drop in cholesterol linoleate and a rise in cholesterol palmitate post-operatively.

The turnover of the plasma cholesterol *in vivo* has been studied using both acetate and mevalonate as cholesterol precursors. When acetate was the cholesterol precursor and the $^{14}CO_2$ was collected, no significant difference emerged between the $^{14}CO_2$ evolution data from the intact and ovariectomized animals. This suggested that in the rat, ovariectomy did not influence the "acetate pool size" and consequently results of studies on conversion of acetate to cholesterol were probably not complicated by a change in this "acetate pool" and could be used as a relative measure of the "rates of synthesis". The turnover of the plasma cholesterol in intact and ovariectomized rats is shown in Fig. 3. When acetate was the cholesterol precursor there was a greater incorporation into plasma cholesterol in the case of intact female rats than in the ovariectomized rats (Fillios, Kaplan, Martin & Stare, 1958; Boyd, 1961) suggesting that the rate of cholesterol synthesis was increased. Since the average slope of the specific activity/time course curves was greater in the case of the intact animals then presumably the rate of degradation (or excretion) was also increased (Boyd, 1961).

When the plasma cholesterol turnover experiment was attempted using mevalonate as the precursor it was found that the rate of cholesterol synthesis as measured by the percentage incorporation of isotope into the plasma cholesterol was not significantly different between the ovariectomized and intact female rats. This suggests that endogenous oestrogens may influence cholesterol synthesis at a point between acetate and mevalonate. Oestrogenic hormones appear to influence cholesterol metabolism in at least two ways. There is an effect on the

Table 1. *Distribution of fatty acids in cholesteryl esters of plasma*

Species	Study	No. of subjects	No. of observations	Plasma total cholesterol (mg/100ml)	Plasma cholesterol ester fatty acid composition							
					14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Rat	Intact female	11	3	77	3	6	11	1	15	44	3	17
Rat	Ovariectomized female	17	4	92	1	14	14	4	13	41	3	10
Rat	Intact male	12	3	68								
Rat	Males treated with hexoestrol (50 μg./day)	12	3	43								
Rat	Intact male	8	2	84	2	8	10	2	20	37	7	14
Rat	Thyroidectomized male	8	2	89	2	4	14	4	21	40	3	12
Rat	Male (fed)	10	3	71	1	12	10	3	12	38	10	14
Rat	Male (24-hr. fast)	10	3	68	1	8	8	5	14	29	14	21
Rat	Male (48-hr. fast)	10	3	77	2	8	7	4	10	21	11	37
Human	Intact males	28	6	243	3	17	5	2	20	44	2	7
Human	Males treated with ethinyloestradiol (200 μg./day)	17	4	209	1	28	7	2	24	27	3	8
Human	Intact males	16	5	248	1	10	4	2	26	51	1	4
Human	Males treated with 3,5,3′,5′-tetraiodo-D-thyronine (DT_4)	15	4	193	1	14	10	6	22	39	1	7

biosynthetic mechanism and also an influence on the rate of degradation or excretion.

Experiments have been performed labelling the plasma cholesterol (and of course liver cholesterol) using [2-^{14}C]-mevalonate and after 24 hr. the bile acids have been isolated from the small intestine as described previously. The results of these studies have not as yet produced significant differences between the intact and ovariectomized female rats.

Studies have been made on the phagocytic activity of the reticuloendothelial system using the carbon clearance method described previously. Thus when the endogenous oestrogen secretions are deleted it seems that there is a small but significant decrease in the RES phagocytic activity in the female rat.

Exogenous oestrogens

A wide range of oestrogenic substances have been studied for their effects on cholesterol metabolism (Drill & Riegel, 1958).

In the human, the administration of oestrogenic substances at certain dosages produces a depression in the plasma cholesterol concentration. The depression in plasma cholesterol produced by oestrogens is accomplished by a diminution in the plasma β-lipoprotein with a concomitant elevation in the plasma α-lipoproteins (Barr, Russ & Eder, 1952; Oliver & Boyd, 1959). The dosage of oestrogen necessary to produce this effect on the plasma cholesterol level is in excess of the threshold oestrogenic dosage and this raised the possibility of producing biologically (or chemically) substances in which the oestrogenic potency was minimal and yet the plasma cholesterol depressant effects might be maintained.

This presupposed that the two effects were, to a certain extent, dissociable. A number of laboratories, including our own, conducted studies on the relative potencies of various oestrogens in different biological systems in an attempt to compare and contrast these biological potencies. A number of interesting compounds emerged from these studies, but unfortunately when tested exhaustively it was established (at least in the human) that in general the minimum dosage of oestrogen necessary to produce an effect on lipid metabolism exceeded the threshold oestrogenic dose. Also when the oestrogen molecule was chemically modified in such a way that its oestrogenic potency was virtually nil the plasma lipid effect also disappeared. Table 2 is a summary of a few of the oestrogens studied for their effects on cholesterol metabolism.

It was desirable to establish whether the cholesteryl ester fatty acid pattern was influenced by the administration of oestrogens. This seemed of importance, because if oestrogens accelerate the turnover of cholesterol then it might prove interesting to establish whether certain cholesteryl esters "turned over" at a more rapid rate than others. The cholesteryl esters were isolated from the plasma of subjects before and

during the administration of large doses of oestrogenic substances (200 μg. ethinyl oestradiol/day) and the fatty acid components of the cholesteryl esters determined as discussed previously. Table 1 shows the distribution of fatty acids in the cholesteryl esters in these subjects. The most dramatic change upon oestrogen administration seems to be the decrease in concentration of the cholesteryl linoleate with a marked increase in cholesteryl oleate and palmitate (Boyd, 1962).

Similar results have been obtained in oestrogen-treated male rats. When the synthetic oestrogen, hexoestrol, is administered to male rats there is a marked drop in the plasma cholesteryl ester fraction, the major drop being in the cholesterol linoleate fraction. This is also shown in Table 1.

The turnover of the plasma cholesterol in normal and hexoestrol-treated male rats has been studied using plasma cholesterol labelled from [1-^{14}C]-acetate and from [2-^{14}C]-mevalonate. The experiments employing acetate as the precursor demonstrated that the "acetate pool size" in oestrogen-treated rats was not different from that occurring in intact, untreated rats as deduced from the $^{14}CO_2$ evolution curves of the type shown in Fig. 1. When the specific activity of the plasma cholesterol of normal and hexoestrol treated rats was compared it was shown that the rate of disappearance of cholesterol from the plasma of the oestrogen-treated animals exceeded that found in normal animals. Typical plasma cholesterol turnover curves are shown in Fig. 3. These

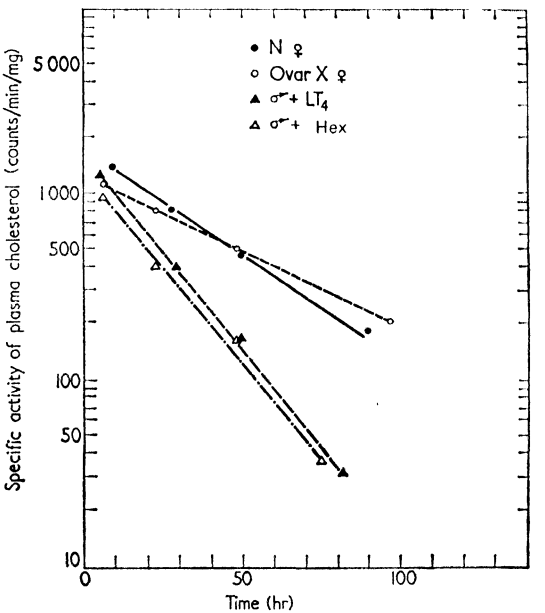


Fig. 3. The rate of disappearance of plasma cholesterol from rats previously injected with [1-^{14}C]-acetate. ● normal females; ○ ovariectomized females; ▲ hyperthyroid males; △ hexoestrol treated males.

experiments suggest that the administration of a potent oestrogen at high dosage to the male rat accelerates the rate of excretion or degradation of the plasma cholesterol.

Studies were made as described previously on the rate of degradation of cholesterol to bile acids using the administration of [2-^{14}C]-mevalonate 24 hr. prior to killing. The bile acids were isolated from the small intestine and the specific activity compared with the specific activity of the liver cholesterol. The results of these studies showed that the rate of degradation of cholesterol to bile acids in the male rat treated with a synthetic oestrogen was not significantly different from that found in the untreated rat.

Studies were performed on the phagocytic activity of the reticuloendothelial system of the male rat treated with a variety of oestrogens. It was found that oestrogenic substances at dose levels in excess of the calculated oestrogenic dose (for the female rat) produced an enhancement of the phagocytic activity of the RES, as shown in Fig. 4.

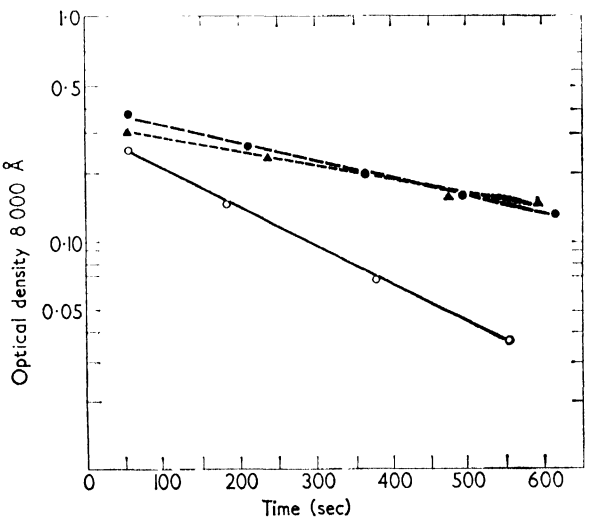


Fig. 4. The rate of disappearance of particulate carbon from the plasma of ● normal male rats; ○ hexoestrol-treated rats; ▲ hypothyroid rats.

Oestrogens may influence cholesterol metabolism through activation of the RES but the dosage of oestrogen required to accomplish this is much in excess of the calculated physiologically available hormone, as shown in Table 2.

These studies on the biological effects of oestrogens in male rats and humans present a problem in attempting to speculate on the possible mode of action of the oestrogens in cholesterol metabolism. The effect of oestrogen administration on plasma cholesterol synthesis and turnover poses the question as to whether the normal removal of lipoproteins or cholesterol from plasma is mediated through the RES

Table 2. *Effect of various oestrogens on plasma cholesterol and on RES*

OESTROGEN		HUMAN		RAT		
Systematic name	Trivial name	Approximate oestrogenic dose (female)	Approximate dose affecting plasma cholesterol concentration	Approximate oestrogenic dose (female)	Approximate dose affecting plasma cholesterol (male)	Approximate dose affecting RES (male)
		μg.	μg.	μg.	μg.	μg.
Oestra-1:3:5(10)-triene-3:17β-diol	oestradiol-17β	200–500	25,000	20	50–100	50
17α-Ethinyloestra-1:3:-5(10)-triene-3:17β-diol	ethinyl oestradiol	10–50	100–200	—	20–100	50
1-Methyl-17-ethinyl oestradiol	—	>10,000	No effect at 10,000	>400	—	—
3-Hydroxyoestra-1:3:-5(10)-trien-17-one	oestrone	1000–5000	50,000	—	*ca.* 200	200
3-Hydroxy-4-methyl-1:3:5(10)-trien-17-one	4-methyloestrone	>20,000	No effect at 10,000	500	>500	—
3:17β-Dihydroxyoestra-1:3:5(10)-trien-16-one	16-oxooestradiol	—	—	15	*ca.* 100	200
Oestra-1:3:5(10)-triene-3:16α:17β-triol	oestriol	100–500	100,000	15	100–200	—
	hexoestrol	200–2000	60,000	—	10–50	50
	stilboestrol	100–1000	40,000	—	10–50	50

system. If this is the normal mechanism for the removal of plasma cholesterol then it is possible that the oestrogen activation of the RES may be the physiological mechanism by which oestrogens influence the plasma cholesterol turnover and concentration. The experimental evidence, however, suggests that the dosage of most oestrogens necessary to produce this effect is greatly in excess of the predicted physiological concentration.

The very marked changes observed in the composition of the cholesteryl esters in tissues and body fluids of animals treated with oestrogen at high dosage suggest that the mechanism of action of oestrogens on cholesterol metabolism may be exceedingly complex.

THYROID

Effects of thyroidectomy

In both the human and the rat the removal of the thyroid gland is accompanied by an elevation in the plasma cholesterol concentration but the degree of elevation of the plasma cholesterol is much greater in the human than in the rat (Boyd, 1961).

The rise in plasma cholesterol concentration after thyroidectomy is accompanied by an increase in the plasma β-lipoprotein in the rat and in the human.

The plasma cholesteryl ester composition is not significantly affected by thyroidectomy in the case of the rat although the period of observation has as yet been quite short, as shown in Table 1. There are no published observations (as far as the author is aware) of changes in the plasma cholesteryl ester-fatty acid composition in humans before and after thyroidectomy.

The turnover of the plasma cholesteryl *in vivo* has been studied in intact and thyroidectomized rats using acetate or mevalonate as the cholesterol precursors. It was found that the estimated "acetate pool size" using the expired CO_2 method described previously was only slightly affected by thyroidectomy. The extrapolated value from the plasma cholesterol specific activity curves indicates that the "rate of synthesis" of cholesterol from acetate is lower in hypothyroid rats (*cf.* Karp & Stetten, 1949; Boyd, 1961; Fletcher & Myant, 1960). The curves also show that the biological survival time is significantly extended. This suggests that the rate of cholesterol degradation or excretion is lower in the absence of the circulating thyroid hormone. When plasma cholesterol synthesis has been studied using mevalonate as the cholesterol precursor the biological half-life has been shown to be increased, but the rate of synthesis from mevalonate to cholesterol appears to be unaffected by the thyroidal status (Fletcher & Myant, 1958).

In studies in which the plasma cholesterol (and liver cholesterol) were labelled *in vivo* using [2-^{14}C]-mevalonate it has been found that the rate of conversion of cholesterol to bile acids during the 24 hr. after

the administration of mevalonate is greater in the euthyroid than in the hypothyroid animal (see Fig. 2). These results are in accord with Eriksson (1957) and suggest that the conversion of cholesterol to bile acids is accelerated by the presence of the circulating thyroid hormone.

Studies have been made on the phagocytic activity of the RES of male rats using the carbon clearance method. The results appear to indicate that in the case of the hypothyroid animal the phagocytic activity of the RES is diminished. This suggests that when the endogenous thyroidal secretions are deleted there is a significant decrease in the RES activity (see Fig. 4).

Exogenous thyroid hormones

A large number of substances have been produced which exhibit "thyroxine-like" activity in the intact animal. The administration of L-thyroxine results in an increase in cellular oxygen consumption rate, heart rate and stimulation of many biosynthetic processes. Although this hormone produces many apparently different effects it is not known whether these effects are mediated through dependent or independent mechanisms. It seemed possible that by varying the structure of the thyroid hormone it might be possible to achieve molecules in which certain effects of the hormone might be more widely discriminated than in the parent structure. A search was made for a "thyroxine analogue" capable of influencing cholesterol metabolism, after the manner of thyroxine, but with minimal effects on "metabolic stimulation". A number of these analogues were tested in various systems and are shown in Table 3 and it will be seen that the various derivatives of

Table 3. *Effects of thyroactive compounds*

Thyroactive compound	Trivial abbreviation	Relative effect on cholesterol metabolism	Relative effect on heart rate	Cholesterol heart rate ratio
3,5,3′,5′-tetraiodo-L-thyronine	LT_4	100	100	1·0
3,5,3′,5′-tetraiodo-D-thyronine	DT_4	10	2·0	5·0
3,5,3′,5′-tetraiodothyropropionic acid	tetraprop.	10·0	10·0	1·0
3,5,3′,5′-tetraiodothyroacetic acid	tetrac	2·0	1·0	2·0
3,5,3′,5′-tetraiodothyroformic acid	T_4F	1·0	0·5	2·0
3,5,3′-triiodo-L-thyronine	LT_3	300	500	0·6
3,5,3′-triiodo-D-thyronine	DT_3	150	20	7·5
3,5,3′-triiodothyropropionic acid	triprop.	20·0	10·0	2·0
3,5,3′-triiodothyroacetic acid	triac	30	20	1·5
3,5,3′-triiodothyroformic acid	T_3F	1·0	0·5	2·0
3,5-diiodo-L-thyronine	LT_2	3·0	10	0·3
3,5-diiodo-D-thyronine	DT_2	2·0	0·4	5·0
3,5-diiodothyroacetic acid	diac	2·0	0·4	5·0
3,5-diiodothyroformic acid	T_2F	—	0·05	—

thyroxine do appear to behave in different ways. A number of these thyroxine analogues were tested in various species but this discussion will be restricted to the effects of the analogues in the rat.

Studies were made of the changes in oxygen consumption rate, hea rate, etc., and various aspects of cholesterol metabolism in order t study the possible differential effects of the analogues.

In studies involving the thyroid hormone and cholesterol metaboli it is important to note that in all quantitative studies there is a marke change in the response of animals at different hormone dose levels. F example, the parent hormone L-thyroxine exerts opposite effects on t "rate of cholesterol synthesis" at different dose levels. When admin stered to euthyroid rats at low dosage level it accelerates cholester synthesis (and oxygen consumption rate, heart rate, etc.) while a higher dose levels (i.e. in the thyrotoxic region) it can depress chole terol synthesis while still maintaining the elevated oxygen consumptio rate and heart rate.

The administration of thyroxine analogues to hypothyroid or euth roid human subjects always produces a decrease in the plasma chole terol concentration and this is usually due to a decrease in the plasm β-lipoprotein concentration (Boyd & Oliver, 1960). Conversely, in t rat it is not always possible to demonstrate a decrease in the plasm cholesterol concentration upon administration of a thyroactive drug t a normal or hypothyroid animal, but if the rat is rendered mildly hype cholesterolaemic (plasma cholesterol concentration elevated to 30 400 mg./100 ml./plasma) it is possible to demonstrate that the adm n stration of thyroxine or a thyroxine analogue produces a decrease in t plasma cholesterol concentration.

The administration of a thyroid hormone to humans produces change in the plasma ester cholesterol-fatty acid composition but t changes are uniform and not as dramatic as in certain other situation A typical result obtained upon the administration of D-thyroxine t 5 human subjects (6 mg./man/day) is shown in Table 1.

The administration of the thyroid hormone to normal rats results i an elevation of the oxygen consumption rate, heart rate, etc., provide the dosage of thyroxine or thyroxine analogue is large enough. If t plasma cholesterol turnover is measured as discussed previously, the it is possible to show that the mildly hyperthyroid rat has an accelerate rate of cholesterol turnover. This suggests that the rate of degradatic (or excretion) of the plasma cholesterol is increased. An example of th phenomenon is shown in Fig. 3 in which the plasma cholesterol w labelled using mevalonate.

The rate of conversion of cholesterol to bile acids has been studied i rats by the technique of injecting mevalonate and killing the anima 24 hr. later. Thereafter the specific activities of the liver cholesterol ar the small intestinal bile acids have been determined. The resul obtained suggest that in the hyperthyroid male rat the conversion cholesterol to bile acids can be elevated above normal. It should b emphasized that as in the case of cholesterol synthesis, the result suc as in Fig. 5 is only obtained in the "mildly hyperthyroid rat" becau

in a number of instances in which the animals have been "thyrotoxic" the rate of conversion of cholesterol to bile acids by this technique has been decreased below normal.

Studies have been made on the phagocytic activity of the RES in euthyroid and mildly hyperthyroid rats using the carbon clearance

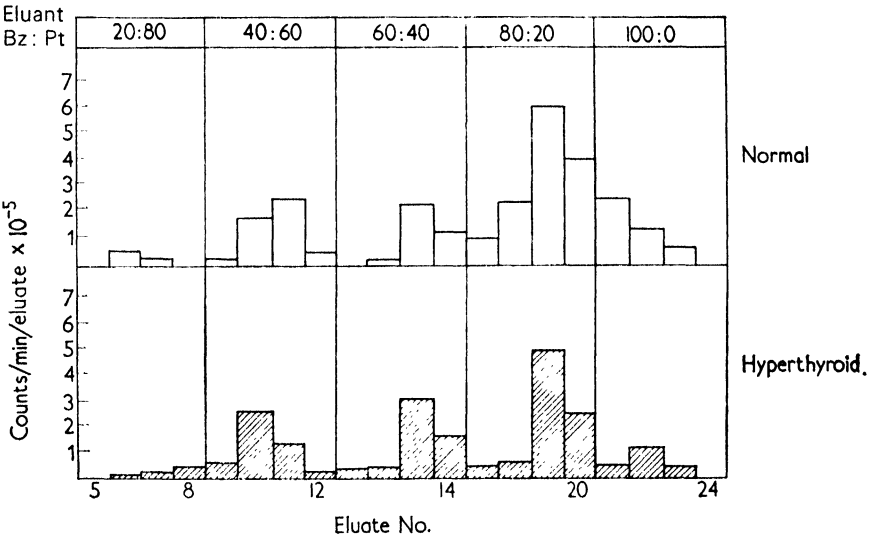


Fig. 5. The chromatographic separation of the small intestinal bile acids using the method of Matschiner *et al.* (1957) which employs benzene: petrol ether :: 70% acetic acid. The open histograms are from normal animals and the hatched histograms are from hyperthyroid animals which were injected previously with [2-^{14}C]-mevalonate.

method. These studies have indicated that in the mildly hyperthyroid rat the phagocytic activity of the RES is little affected but does tend towards increased activity. Once again the production of "thyrotoxic states" annuls this effect.

A large number of substances have been synthesized of the type shown in Table 3 in an attempt to produce a variant on the thyroxine molecule capable of producing an acceleration of cholesterol turnover but devoid of "metabolic stimulant" activity. These studies have been successful to a very limited degree, because while many substances have been produced which exhibit low "metabolic stimulant" effects, these substances have tended to be low or ineffective towards lipid metabolism. As yet it has been impossible to obtain a substance related to thyroxine which can influence cholesterol metabolism while being devoid of metabolic stimulant activity.

The problem of the mode of action of thyroxine and the turnover of cholesterol remains unsolved and since it has been impossible to dissociate the metabolic stimulant effects from the cholesterol effects, it may be that they are dependent functions. If this is so, then it is interesting

to try to speculate on the mode of action. The thyroid hormone appears to accelerate the plasma cholesterol degradation or excretion. The evidence presented suggests that the hormone can increase the rate of degradation of cholesterol to bile acids. This process is both an oxygenation and oxidation reaction and it may be that the turnover of cholesterol through these reactions is elevated as part of a general increase in cellular oxidative metabolism. As yet all the steps in the degradation of cholesterol to bile acids have not reached the same degree of elucidation as those involved in cholesterol synthesis (Bergstrom *et al.*, 1960). It is hoped that as we learn more about the pathway of cholesterol degradation the possible mode of action of thyroxine in this sequence may be clarified.

FASTING

Effects of fasting on cholesterol metabolism

In studies on the fine control of metabolism through the endocrine system it is desirable to compare endocrine influences with some comparable physiological constraint such as withholding food, bearing in mind that the latter stress may produce effects mediated through the endocrine system. When a rat is fasted for 24–48 hr. there are only minor changes in the plasma cholesterol concentration and in the plasma lipoprotein patterns.

When animals are fasted and injected with labelled acetate, it is possible to show that the "pool size" of acetate and/or the turnover of acetate is modified in the fasted animals as shown in Fig. 6. The rate of evolution of CO_2 during the primary phase of oxidative metabolism is elevated in the fasted animal and differences can also be detected in the rate of evolution of CO_2 in the secondary phase of oxidative metabolism.

When the rat is fasted and injected with [1-^{14}C]-acetate it has been shown that cholesterol synthesis drops to a very low level so that the amount of labelled cholesterol appearing in plasma is low. However, when [2-^{14}C]-mevalonate is used as the cholesterol precursor, cholesterol synthesis proceeds normally in the fasted animal and this has been explained by Bucher, Overath & Lynen (1960) as due to the fact that it is the β-hydroxy-methyl-glutaryl-CoA reductase which is decreased in activity upon fasting. Consequently, the administration of mevalonate to the fasted animal bypasses this block in metabolism and so the plasma cholesterol in the fasted animal is labelled to an equal degree to that found in the fed animal.

If the cholesteryl esters are isolated from rat plasma and the cholesterol ester fatty acid composition examined there are very marked changes in the plasma cholesteryl esters. The results of an experiment of this type in which male rats were fasted are shown in Table 1. It will be noted that fasting produces a decrease in the cholesteryl linoleate and an elevation in the cholesteryl arachidonate.

When the biological survival time of the plasma cholesterol from mevalonate-treated animals is compared between fasted and fed animals it is found that the half-life of the plasma cholesterol is extended in the fasted animals. This suggests that the rate of cholesterol degradation or excretion is decreased during fasting.

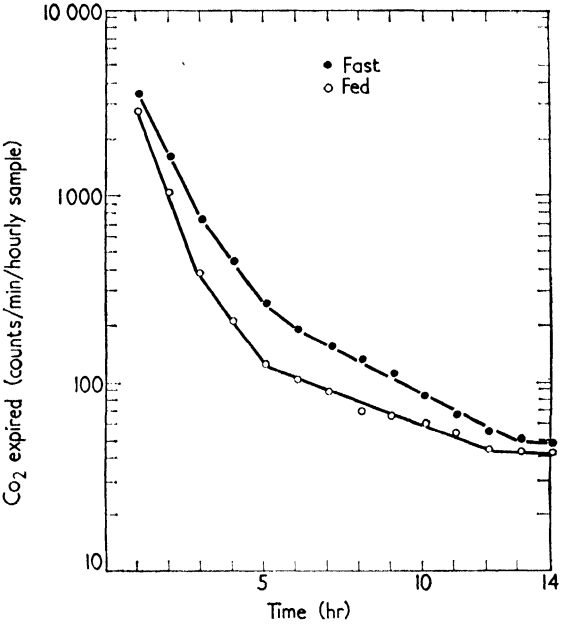


Fig. 6. The $^{14}CO_2$ expired by male rats injected intraperitoneally with [1-^{14}C]-acetate at zero time. ● fasted animals; ○ fed animals.

When fasted animals are injected with [2-^{14}C]-mevalonate and allowed to survive for 24 hr. and the specific activity of the small intestinal bile acids determined, it is found that there is only a slight difference between the specific activities of the acids isolated from the fasted and fed animals suggesting that the rate of degradation is little influenced by fasting, as shown in Fig. 7.

DISCUSSION

The circulating cholesterol is largely synthesized in the liver and discharged into plasma bound to lipoproteins in which it is present in the unesterified and various esterified forms. The plasma cholesterol and cholesteryl esters equilibrate with tissue cholesterol and cholesteryl esters, and some tissues extract cholesterol from plasma for their cellular requirements, despite their having the necessary enzymes to effect cholesterol synthesis. The "plasma cholesterol pool" equilibrates in a differential fashion with a number of tissues and consequently measurement of the disappearance of sterol from the plasma compartment is a complex system to analyse. The plasma cholesterol pool

reflects the net result of a series of reactions which are donating sterol to the pool whilst others are removing sterol. In addition the cholesteryl esters present in plasma can be hydrolyzed and synthesized within this

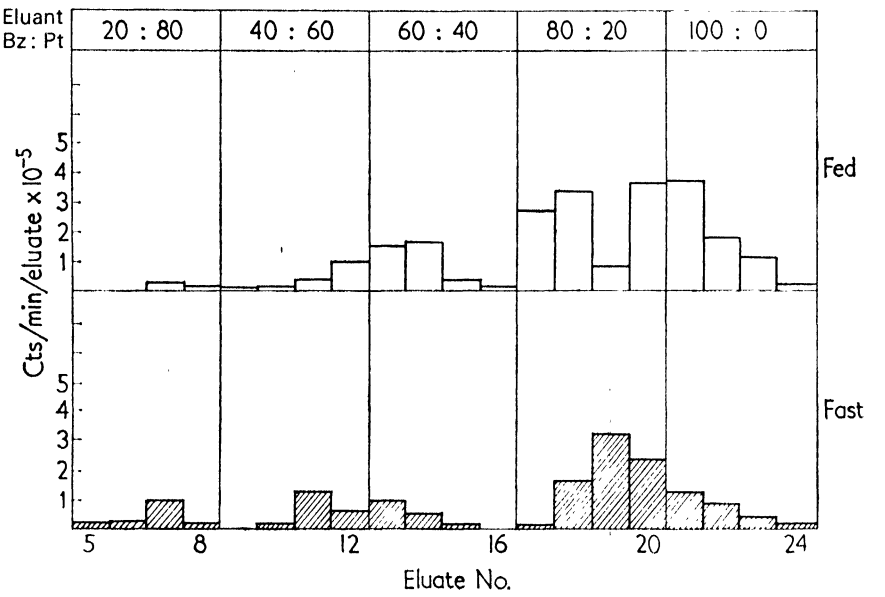


Fig. 7. The chromatographic separation of the small intestinal bile acids using the method of Matschiner *et al.* (1957) which employs benzene: petrol ether :: 70% acetic acid. The open histograms are from normal fed animals and the hatched histograms are from normal fasted animals which were injected previously with [2-^{14}C]-mevalonate.

fluid compartment and this complicates further the interpretation of cholesteryl ester turnover patterns. Examples of various factors which influence the cholesteryl ester fatty acid pattern have been given in this paper and elsewhere.

We have methods which permit us to obtain information on the "rate of synthesis of the plasma cholesterol" by the use of isotopically labelled precursors. This is possible because there is a single route leading to cholesterol synthesis and a predominant organ—the liver—involved in the synthesis of the plasma cholesterol.

The problem in studying the withdrawal of cholesterol from the plasma pool is much more complicated, due to the possible participation of a large number of organs and also a series of biochemical degradation reactions involving cholesterol. Attempts have been made in this paper to obtain a semi-quantitative assessment of the rate of breakdown of cholesterol to bile acids. This approach is complicated by the fact that the intestinal flora can modify the large intestinal bile acids which are reabsorbed and reexcreted. The bile acids are known to exert a feedback control on cholesterol synthesis and degradation. It is possible to obtain a measure of the rate of cholesterol degradation to bile acids by

labelling the "plasma-liver pool" of sterol with a suitable cholesterol precursor and determining of the activity of the bile acids in the small intestine after a short time interval.

It is possible to compare effects on the plasma cholesterol biological survival time with the semi-quantitative assessment of this single, but quantitatively important, cholesterol degradation route. These methods have been applied to the study of the mode of action of a few endocrinological factors in the control of cholesterol metabolism.

Using acetate as the cholesterol precursor the effect of various hormones on cholesterol synthesis has been demonstrated. In some of these cases no effect is observed when mevalonate is used as the precursor. This is evidence that the point at which certain hormones influence cholesterol metabolism lies between acetate and mevalonate. From observations made on the rates of cholesterol synthesis using cell-free liver extracts from the tissues of animals fasted (Bucher *et al.*, 1960), or "cholesterol fed" (Gould & Popjak, 1957), it has been shown that synthesis from mevalonate is unimpaired. One sensitive enzyme system is the β-hydroxyl-β-methyl-glutarate reductase shown to be affected by fasting (Bucher *et al.*, 1960), and some hormones may affect cholesterol metabolism through this enzyme system. As yet, no hormone has been shown to exert an effect on cholesterol synthesis *in vitro*. Consequently, most studies are modifications of enzymic sequences in tissues obtained from animals previously treated with a hormone. Interpretation is then difficult because the effect could be relatively remote; for example, it could be due to the hormone altering one or more enzyme concentrations.

From the experiments performed on the biological half-life of the plasma cholesterol taken in conjunction with the experiments on the conversion of cholesterol to intestinal bile acids, it seems that oestrogens at the dose levels used in these experiments had little effect on the conversion of cholesterol to bile acids. On the other hand, the experiments confirmed that the mildly hyperthyroid rat appeared to have an elevated rate of conversion of cholesterol to bile acids (Eriksson, 1957) suggesting that the thyroid hormones accelerate this oxidative catabolic transformation.

The precise point at which thyroxine acts is not yet known, because the hormone has little, if any, effect *in vitro*. In this transformation to bile acids as in all other reactions involving thyroxine there is a "lag phase" between the hormone administration and any effect and it is, therefore, impossible to establish the mechanism of the hormonal activation. In the studies in which cholesterol has been fed to rats to elevate the liver sterol concentration artificially it has been shown that thyroxine treatment of these animals results in a general decrease in cholesterol esters present in this organ. This is similar to the effect of a thyroxine analogue (DT_4) on the cholesteryl ester pattern in human plasma.

There is as yet no evidence for the existence of an oestrogen analogue in which the effects on cholesterol metabolism are retained without oestrogenic side effects. It is possible within a series of oestrogenic compounds to detect discrimination of effects obtained in different biological parameters but no compound has emerged which will affect cholesterol metabolism without exhibiting significant oestrogenicity.

In the same way a number of analogues of thyroxine have been examined in different biological systems. While some of these analogues appear to have a more selective effect on cholesterol metabolism than on general metabolic stimulation no analogue has been produced capable of exerting an effect on cholesterol metabolism without significantly influencing oxidative metabolism.

It is interesting to contrast the effects of fasting on cholesterol metabolism with some of the endocrine effects. The acetate pool size of fasted animals appears to be altered but there is little change in the concentration of cholesterol in the plasma of the rat. The hepatic cholesterol synthesis from acetate is greatly inhibited while the synthesis from mevalonate is unaffected. This suggests that the block in cholesterol synthesis may occur as suggested by Bucher *et al.* (1960) at the reductase enzyme. The cholesteryl ester fatty acid pattern changes a great deal. The cholesteryl linoleate is reduced and the cholesteryl arachidonate is elevated, while the rate of cholesterol degradation to bile acids is little changed or decreased. Cholesterol is an integral part of cell membranes and sub-cellular particle membranes and so in studies on cholesterol degradation we may be measuring (in part) the rate at which certain sub-cellular entities such as mitochondria are being produced and degraded.

Many of the oxidative reactions in cholesterol degradation are associated with mitochondria which are rich in fatty acids of the poly-unsaturated-methylene-interrupted type. It is known that the turnover of cholesterol can be influenced by dietary factors such as the fatty acid composition and especially the linoleic acid content of the diet (Avigan & Steinberg, 1958; Boyd & Mawer, 1959). Linoleic acid appears to be one of the acids selectively esterified to cholesterol, as in most tissues and body fluids under normal dietary conditions cholesteryl linoleate is present in high concentration. It is tempting to suggest that since cholesterol appears to esterify selectively to linoleic acid and as the latter acid can be shown to influence cholesterol turnover, the substrate for metabolism might be cholesteryl linoleate. Poly-unsaturated fatty acids of the linoleate type are susceptible to oxidation to yield hydroperoxides and a molecule of this sort might conceivably donate an oxygen atom to an allylic hydrogen (such as occurs at carbon 7) in the sterol structure to produce a hydroxylated analogue of cholesterol ester. Hydroxyesters have been isolated from plasma and tissues and these substances can be hydrolysed by cholesteryl esterases to yield 7α-hydroxycholesterol (Boyd & Mawer, 1959).

The factors which dictate the portioning of cholesterol between the plasma and the various tissues are not fully understood. This makes the studies on the biological half-life of plasma cholesterol difficult to interpret unless these observations are coupled to independent assessment of cholesterol disappearance to defined tissues or products.

A number of hormones have been shown to affect the concentration or turnover of sterol in various tissues and body fluids. In general, endocrinological effects on cholesterol metabolism are quantitatively of a lower order of magnitude than certain other physiological effects such as dietary changes or fasting. This means that one must view hormone studies in perspective and not over-emphasize their importance in this context. The endocrinological control of cholesterol metabolism should be looked upon as a fine regulator of complex processes superimposed upon primitive control mechanisms.

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DISCUSSION

S. J. Folley: Have you made any recent progress in finding thyroxine analogues which effect cholesterol without affecting oxidative metabolism? In this connection, sometime ago we investigated another phase of lipid metabolism, the secretion of fat by the mammary gland of the cow. We examined one of your compounds, BT3, which I think had the

highest ratio of activity on cholesterol metabolism to heart rate, in the hope that we could find that it would increase the milk fat percentage without increasing the general metabolism of the animal. In order to get any effect on the milk fat percentage we had to give such a dose that there was a marked increase in thyroid metabolism.

G. S. Boyd: We have not found any other compound in this series in which we can dissociate the lipid (the cholesterol) effect from the metabolic stimulant effect.

D. Steinberg: My first question relates to the assumption that the counts appearing in bile acid over the 8-hour period after mevalonate give you a fair reflection of the cholesterol breakdown. Was the curve for the changing specific radioactivity of liver and/or plasma cholesterol during that 8-hour period measured in the animals treated in different ways?

G. S. Boyd: Yes. The strange thing is that even in hypothyroid animals we were impressed by the fact that the mevalonate conversions to cholesterol in the liver, plasma and small intestinal pool are so similar. We are, however, dealing with pools that are quite similar, and which have apparently come into equilibrium, but if we wait too long to achieve this (and we cannot wait beyond 8 hours) then bile acids have gone into the large intestine.

D. Steinberg: Some years ago we studied the effects in rats of dietary fat on the rate of cholesterol biosynthesis in the liver. The animals were on a diet supplemented with 20% by weight of fat. (How much fat was in your diet?) We found that feeding a corn-oil supplement, for long enough to ensure that the rats were in a steady state with regard to liver and plasma cholesterol, caused a marked increase in the rate of cholesterol synthesis, as measured either by incorporation of acetate or of tritiated water from the body pool of water. Since the animals were in a steady state by this time we could infer (although we did not measure it) that the rate of cholesterol breakdown must likewise have been accelerated to match that of cholesterol synthesis. Your results suggest there was no effect on the rate of cholesterol breakdown. Would you please comment on this?

G. S. Boyd: Our animals were on a diet in which 10% of the calories were derived from the fat. They were on this diet for about 6 weeks before we injected them. The actual percentage incorporation of mevalonate into the sterol does not matter in our calculations because we were measuring the total activity in the bile acids divided by the total activity in the bile acids, plus liver, plus plasma and small intestinal fluid from pools, over a number of hours.

INFLUENCE OF THYROID AND ADRENOCORTICOTROPHIC HORMONES AND OF GENETIC FACTORS ON CHOLESTEROL METABOLISM IN THE RAT

By G. FEUER

Medical Research Council, Neuropsychiatric Research Unit, Carshalton, Surrey, England

SELECTIVE breeding of albino rats for different behavioural characteristics resulted in two strains (Broadhurst, 1960) which differed in thyroid activity and in cholesterol metabolism (Feuer, 1963). The Reactive strain showed slight hypothyroidism and raised cholesterol level in comparison with the Non-reactive strain. Cholesterol levels in these animals were higher than the values generally given in the literature. (Reactive rats: 112·78 ± 2·45 S.E. mg/100 ml serum, Non-reactive rats: 97·3 ± 3·23; literature values: 28–76 Boyd, 1942.) In the Reactive rats decreased adrenal cortical activity was also found. It was therefore decided to investigate whether the thyroid and adrenal functions were related to the serum cholesterol level.

One group from both strains was treated with thyroxine (5 μg DL-thyroxine, British Drug Houses, s.c./100 g body weight), other groups with adrenocorticotrophic hormone (ACTH) (2 i.u. Cortrophin-Zn Organon s.c./100 g body weight, from 5 days of age until behavioural test). After thyroxine treatment the higher cholesterol level in the Reactive strain was significantly decreased (101·71 ± 4·53 mg/100 ml serum; $P < 0{\cdot}01$).

ACTH treatment was also followed by a lower serum cholesterol level (Reactive rats 98·6 ± 3·22 mg/100 ml serum, Non-reactive rats 88·0 ± 4·16 mg/100 ml). Similar observations were previously described in hypothyroid patients and patients treated with ACTH (Skanse, Studnitz & Skoog, 1959) and in rabbits (Komarova, 1960).

When rats from the Carshalton strain with comparatively low cholesterol content were given ACTH (1–2 i.u., i.p./100 g body weight), a slight increase in cholesterol level was obtained within 1–3 hours: it returned to normal or below the resting value approximately 4 hours after administration. No change occurred in the Reactive rats, but in some of the Non-reactive animals a slight (5–8%) change in cholesterol level was found. This apparent increase of the serum cholesterol level

was associated with a decrease of the adrenal cholesterol. It is concluded that ACTH may decrease the cholesterol as well as the corticosteroid content of the adrenal gland.

The serum cholesterol level showed significant differences in different strains of albino rats, and apparent variations in the cholesterol content of the liver and adrenal were found. No difference was observed in the brain cholesterol level. These experiments suggest that cholesterol metabolism depends on genetic factors as well as differences in adrenal activity associated with different sensitivity towards emotional stimuli in the environment.

A grant from the Mental Health Research Fund is gratefully acknowledged.

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DISCUSSION

G. S. Boyd: I think that the differences between species in their reaction to ACTH and cortisone are very interesting. Even within species the reaction to cortisone can be variable. There are almost equal numbers of reports in the literature suggesting that cortisone either elevates or depresses plasma lipids. In the rat you can show a very marked dose response curve using cortisone. I wonder whether with ACTH without altering the dose, you have ever been able to modify the response?

G. Feuer: In some other experiments the ACTH level was doubled and we used long-acting ACTH zinc hydroxyde complex. We found a little higher serum cholesterol level and the response to ACTH, measured by counting hourly the eosinophil cells, was also interesting. In these experiments we found extreme differences between the reactive and the non-reactive rats.

R. Paoletti: If you have measured corticosteroid levels in blood after your long-acting ACTH, have you found that strains of animals differ in the response of their adrenal glands to the ACTH?

G. Feuer: We did not measure the increase in the corticosteroid level after ACTH injection but we assayed the resting corticosteroid level in these animals. In all the strains which we studied we found great differences. Significant differences in corticosteroid level were observed, for example, between the reactive and non-reactive strains which originated from the same parental strain.

according to which plasma lipids are deposited in the vessel wall and (2) the thrombogenic theory of Duguid, according to which small thrombi are deposited on the endothelial surface of the vessels (see Fig. 1). These mural thrombi became incorporated into the vessel wall by

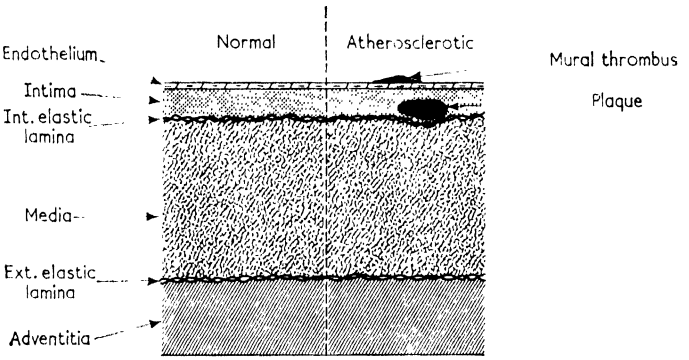


Fig. 1. Diagram of structure of normal and atherosclerotic aorta. The endothelium is a single layer of cells lining the vessel wall. The intima is composed of loose connective tissue; the plaque is situated in the deeper layer of the intima. The media is mainly composed of elastic fibres. The elastic laminae, internal and external, are bands of fused elastic fibres. The adventitia is composed of connective tissue.

overgrowth of the surrounding endothelium, thus giving rise to the atherosclerotic plaque. Hyperlipidaemia has been suggested as the common link, predisposing to the deposition of plasma lipids in the vessel wall, and, at the same time, giving rise to a disturbance of blood coagulation mechanisms, resulting in the development of mural thrombi.

The aim of the present investigation was threefold. Firstly to study the distribution of the major lipid fractions viz. cholesteryl esters, triglycerides and phospholipids in the atherosclerotic plaque (Stage II). Secondly, to compare serum and plaque lipids (particularly fatty acid patterns). Thirdly, to try to form an opinion based on these investigations as to the origin of lipid in the atherosclerotic plaque.

METHODS

Serum was obtained from patients who had previously sustained a myocardial infarction, and the lipoprotein fractions were separated according to the method of Havel, Eder & Bragdon (1958). The plaques were obtained from patients who had died from an acute vascular incident, such as myocardial infarction or cerebral thrombosis.

The major lipid components of serum and plaque viz. cholesteryl esters, triglycerids and phospholipids were separated by silicic acid chromatography (Benzene-Hexane Solvent System). The fatty acid patterns of each of these major components were then obtained by Gas Liquid Chromatography.

For the purposes of histological and histochemical study a portion of

the plaque was fixed in formol-calcium, embedded in gelatin and sectioned at 10 μ on a freezing microtome.

The following stains were used:

(1) Sudan IV—total non-protein bound lipid.
(2) Nile Blue Sulphate (Menschik, 1953)—phospholipid.
(3) Acid Haematein (Baker, 1946)—phospholipids.
(4) Osmium Tetroxide—α naphthylamine (OTAN)—Adams, 1959b. It is claimed that protein-bound phospholipid is shown by this method.
(5) Formol Fuchsin and Picro Ponceau S—elastic and collagen tissue respectively.
(6) Haematoxylin and Eosin—general structures of vessel wall.

RESULTS

The fatty acid patterns of the major lipid fractions of serum lipoprotein and plaques are presented in Tables 1, 2 and 3.

The histochemical study provided information about the distribution of the various lipid fractions, particularly phospholipid in the plaque. The atherosclerotic plaque consisted mainly of extracellular lipid in the form of cholesterol, cholesteryl esters and triglycerides (hydrophobic lipid). Non-protein bound phospholipid, on the other hand, was localized within macrophage cells, which were distributed along the intimal side of the plaque; occasionally phospholipid-containing macrophages were seen embedded in the mass of extracellular lipid. This contrasts with the distribution of protein-bound phospholipid, which Adams (1959) claims to have demonstrated on the medial side of the plaque in its early stages; this is said to be derived from degenerating elastic fibres and to constitute an early stage of the lesion. Macrophage cells containing hydrophobic lipid were also seen, intermingled with the phospholipid-containing macrophages.

DISCUSSION

When comparing the fatty acid patterns of serum lipoproteins and plaques, it would be desirable to have serum and plaque from the same individual but the design of such a study presents considerable difficulty. In a static study such as this one, it would be unwise to draw too definite conclusions from an investigation of one aspect of this problem (viz. fatty acid patterns). It is necessary to take into account the information obtained from this and other forms of study such as histology, immunochemistry and ultracentrifugation of plaque lipoproteins.

From a purely theoretical point of view, plaque lipids might accumulate in one of several ways:

(1) By deposition of total plasma lipids.
(2) By selective deposition of plasma lipids, e.g. specific lipoprotein fractions.
(3) By synthesis of lipid in the vessel wall.

(4) By incorporation of mural thrombi into the vessel wall with subsequent fatty degeneration.
(5) Various combinations of the above.

At the same time lipid may be removed from the vessel wall and this process may be selective.

(*a*) *Cholesteryl ester fatty acid patterns*

It will be seen from Table 1 that there is a fairly close correlation between the fatty acid patterns of plaque and of the various serum lipoprotein fractions. The results are compatible with the view that the

Table 1. *Cholesteryl ester fatty acid pattern**

Fatty acid	Plaque	Serum lipoproteins		
		Sf 20–400	Sf 0–20	HDL 2 + 3
14:0	2·1 (0·8–2·4)	2·0 (1–2·6)	1·6 (0·5–2)	2·6 (1·1–3·5)
16:0	15·2 (11–23)	13 (11–15·4)	10·6 (8·6–12·7)	15·5 (10·6–17·5)
16:1	11·3 (8·9–13·3)	9·2 (7·4–12·0)	7·7 (6·4–10·5)	9·0 (5·8–11·1)
18:0	2·1 (0·5–5·6)	2·6 (2·1–4·7)	1·8 (1–1·7)	2·4 (1·1–5·4)
18:1	21·4 (16–28·3)	22·1 (15–26)	19·0 (13·7–25·4)	18·5 (14·8–18·8)
18:2	38·0 (25–45)	36·0 (28–52)	45·0 (38–55)	42·0 (34·4–52·2)
20:4	5·3 (2·6–7·0)	6·0 (4–9)	5·6 (2·4–9·5)	7·0 (4·4–8·5)

* Each fatty acid is expressed as a percentage of total fatty acids from 14:0 (myristic) to 20:4 (arachidonic).

cholesteryl ester fatty acids of plaque are mainly derived from serum but it is not possible to say whether there is selective deposition of a particular lipoprotein or not. From a quantitative point of view, by far the greater proportion of serum cholesteryl esters (in atherosclerotic patients) are carried in the Sf 0–20 group and it might be inferred from this that plaque cholesteryl esters are mainly derived from this group of serum lipoproteins. Smith (1960) has reached a similar conclusion based on the comparisons of serum and plaque fatty acid patterns.

In order to reach a decision as to whether or not there is selective deposition of a serum lipoprotein fraction, it is necessary to take into account the evidence from immuno-chemical and ultracentrifugal studies of plaque lipoproteins; but here the results are conflicting. Ott, Lohss & Gergely (1958) and Gero, Gergely, Jakab, Szelkely & Virag (1961) using immuno-chemical techniques, did not find α (HDL)

lipoprotein in the plaque whilst Hanig, Shainoff & Lowry (1956), using ultracentrifugal methods, could find no evidence of Sf 0–12 lipoproteins in atherosclerotic plaques. It is obvious that these investigations require to be confirmed or refuted before a definite decision is reached.

(*b*) *Triglyceride fatty acid patterns*

As with cholesteryl ester fatty acid patterns the results are broadly compatible with the view that the plaque fatty acids of triglycerides are mainly derived from serum but it is not possible, on a qualitative

Table 2. *Triglyceride fatty acid pattern*

Fatty acid	Plaque	Serum lipoproteins		
		Sf 20–400	Sf 0–20	HDL 2 + 3
14:0	3·8 (2·0–6·8)	4 (3·4–5·2)	5·0 (3·6–5·7)	5·0 (3·7–6·1)
16:0	25 (16·7–29)	29·0 (24–36)	26·8 (24–33)	25·0 (2–28·4)
16:1	9·4 (7·3–19)	8·7 (7·6–12·6)	10·6 (8–14)	12·2 (9–15·6)
18:0	3·3 (1–5·3)	4·3 (2–6·5)	4·0 (2·6–4·8)	3·6 (2·8–4·1)
18:1	30·6 (23–44)	35·0 (33–41)	32·0 (27–38)	30·0 (24–36)
18:2	9·5 (7·0–12·5)	7·5 (1·5–10)	8·5 (6·5–12)	8·3 (7–12·5)
20:4	1·6 (1–2·1)	2·1 (1·5–4)	2·5 (1·1–4·7)	2·8 (1·4–4·9)

comparison of fatty acid patterns, to say whether there is selective or non-selective deposition.

From the quantitative point of view the greater proportion of serum triglycerides is carried in the Sf 20–400 group and it might be inferred from this that the Sf 20–400 group form the main source of plaque triglyceride fatty acids.

If these "quantitative arguments" are accepted it suggests that the plaque fatty acid patterns should be similar to the serum fatty acid patterns of the Sf 0–20 plus Sf 20–400 groups, for both cholesteryl esters and triglycerides. The results presented in Tables 1 and 2 are compatible with this, as are the results of immunochemical studies.

(*c*) *Phospholipid fatty acid patterns*

It will be noted that there is a fairly close correlation between the fatty acid patterns of the three lipo-protein fractions. This has also been noted by Lindgren & Nichols (1961).

The most noticeable difference between plaque fatty acid and serum fatty acid patterns is the lower amount of linoleic acid in plaque. Boettcher & Van Gent (1961) has reported that, in this type of lesion, sphingomyelin occurs in greater concentration (56·8%) than any other

Table 3. *Phospholipid fatty acid pattern*

Fatty acid	Plaque	Serum lipoproteins		
		Sf 20–400	Sf 0–20	HDL 2 + 3
14:0	1·9 (0·3–3·5)	1·4 (1–1·8)	2·0 (1–6·4)	1·6 (0·7–5·0)
16:0	32 (20–44)	29·5 (22–38)	27·0 (23–30)	27·8 (24–30)
16:1	5·4 (4·1–7·3)	6·5 (3·3–10·4)	5·8 (2·0–9·6)	6·0 (2·7–12·5)
18:0	10·3 (6·6–16)	8·8 (6·0–13·3)	9·8 (6·0–13·5)	9·4 (8·0–12·4)
18:1	16·5 (17·4–20·2)	18·9 (14·6–26·0)	18·3 (13–29)	16·1 (13·7–21·3)
18:2	9·3 (7·1–18·0)	18·5 (12·4–20·0)	18·6 (9·6–27·0)	14·4 (4·4–22)
20:4	9·0 (5·8–12·6)	7·0 (1·4–10·7)	6·8 (2·5–12·5)	9·4 (3–13)

phospholipid fraction; this might account for the low concentration of linoleic acid. Nelson & Freeman (1960) have shown that, in the three lipo protein fractions of serum, lecithin is present in greater concentration than any other phospholipid fraction and that 70% of all serum sphingomyelin is carried in the Sf 0–20 group. If there is selective deposition of this group in atherosclerosis, then it is necessary to postulate, in addition, selective removal of other phospholipid fractions, particularly lecithin, from the vessel wall. Alternatively local synthesis of phospholipid in the vessel wall might be considered or some combination of these three mechanisms.

It is evident from the histological study that cholesterol, cholesteryl esters and triglycerides (mainly extracellular) are dealt with in a different manner from phospholipids (intracellular). The intracellular localization of phospholipid together with evidence from other sources, tend to support the hypothesis of synthesis of phospholipid within the vessel wall, e.g. Day & Fidge (1962) has shown that macrophage cells of rabbit peritoneum can synthesize phospholipid; Day (1962) has also described a somewhat similar distribution of intraphagocytic phospholipid in the experimental lesion in rabbits. The studies of Zilversmit (1959) and Zilversmit & McCandleas (1959) have also demonstrated the synthesis of phospholipid in aorta, in both human and experimental

lesions. Christensen (1961) has studied the rate of transfer of phospholipid and phosphoprotein, using P^{32}, across the intimal surface of the thoracic aorta of the cockerel and concludes that the ratio of phospholipid derived from synthesis to phospholipid derived from the plasma, in the aorta of the stilboestrol-treated cockerels, is 10:1.

There is, therefore, strong circumstantial evidence in favour of phospholipid synthesis in the aortic wall but it has not yet been determined in humans what proportion is derived from deposition from plasma and what proportion is derived from local synthesis.

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DISCUSSION

A. N. Howard: As you pointed out atherosclerosis is a disease of the intima. In your paper you state you have analyzed atherosclerotic plaques. I wonder whether this includes media or is it only the intimal section of the plaque?

T. D. V. Lawrie: The material analyzed was plaque, with as little of the surrounding intima as possible. The medial coat was not included.

A. N. Howard: So the media was, in fact, stripped.

T. D. V. Lawrie: Yes.

A. N. Howard: I wonder also whether you would like to comment on the results which Dr. Elspeth Smith presented last year in Edinburgh, at the Biochemical Society Meeting in June, in which she gave certain data on

the eicosatrienoic acid content of the plaques. As many of you may know, in essential fatty acid deficiency, eicosatrienoic acid accumulates in the plasma and Elspeth Smith, by analyzing only the intima of diseased subjects and comparing it with undiseased intima, found that there was an increase in this abnormal fatty acid in the pathological tissue. I note that in your tables this fatty acid is not described.

T. D. V. Lawrie: In the tables only the major fatty acids are listed. Small amounts of the particular fatty acid to which you refer were found in the fibro-fatty plaque. I understand that Holman regards the presence of this fatty acid as an indication of essential fatty acid deficiency but I am not prepared to speculate on its significance in human atherosclerotic plaques.

Elspeth Smith: I feel I must make some comment since Dr. Howard raised this point. In my studies I investigated the fatty streak. Now Dr. Lawrie has not analyzed this particular type of lesion at all. There is considerable question as to whether the fatty streak, which is the earliest lesion occurring in children and young people, is, in fact, the precursor of the more advanced type of plaque. In the fatty streak you find a very remarkable cholesteryl ester fatty acid pattern which bears practically no resemblance to that found in serum. You get an extremely low linoleic acid and a very high oleic acid content, and you get a high proportion eicosatrienoic acid. If from the same aorta you compare fatty streak with the type 2 lesion which Dr. Lawrie was investigating you find that the lipids in the type 2 lesion resemble the lipids of plasma very much more closely than in the fatty streak. Now this is all very confusing, and indeed I think it strongly suggests that the fatty streak is on a different line of development from the fibrous plaque which is found in older lesions. It also raises very sharply the point of where this cholesteryl ester comes from.

Looking at the fatty streak lipid one certainly would not postulate that it comes from serum. Looking at the advanced type of lesion one might well postulate that it does come from serum. One might say that it is changed when it gets into the vessel wall, but the interesting thing is that the major change appears to be in the most juvenile and presumably youngest type of lesion, where one might think there had been less opportunity for change than in the older type of lesion. I think that really one must be extremely careful still in postulating a serum origin for all the lipids in atherosclerotic plaques.

T. D. V. Lawrie: I think this question of the relationship of the fatty streak to atherosclerosis is still an open one. For reasons, which I mentioned in the paper, one is rather reluctant to accept this as the initial lesion, at least on the present evidence. These are based not merely on biochemical grounds but pathological grounds as well. Furthermore, I understand that electron microscopy studies suggest, and this again is

speculative, that the lipid in the fatty streak is synthesized by cells which appear to be related to muscle cells. Because of this dubious relationship to atherosclerosis, I have not analyzed the fatty streak.

D. E. Bowyer: If one analyzes the plaque of an aorta from a patient aged 60 one finds, for both healthy and diseased intima, cholesteryl ester patterns which are different from these of the plasma, but if one analyzes the media from the same aorta the patterns for cholesteryl esters are similar to those of the plasma. I find mean values of figures for the intima and the media very similar to your own and to Boettcher's for the plaque stage 2 and I wonder if, in fact, the pattern of the media is due to the presence of cholesteryl esters from the plasma whilst the pattern of the intima is very different.

T. D. V. Lawrie: Boettcher analyzed media plus intima and his results are similar to my own analysis of plaque alone. It is not surprising that the fatty acid patterns of cholesteryl ester of media are similar to serum because the medial coat contains vasa vasorum, which, in turn, contain blood. As I mentioned earlier, different workers report different results mainly because they are analyzing different parts of the vessel wall.

P. F. Wilde: On a point of methodology, have you tried the selective polychrome staining technique of Kennedy and Collier? They use mixtures of Solochrome and crystal violet. This was developed as a histological stain and has been extended to cover paper chromatograms.

T. D. V. Lawrie: The only other dye that has been used is a Rodamine 6 G. Using the fluorescence microscope we confirmed the presence of phospholipid droplets within the macrophages.

FATTY ACID MOBILIZATION—MECHANISMS OF REGULATION AND METABOLIC CONSEQUENCES

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INTRODUCTION

Adipose tissue triglycerides constitute the major storage form of oxidizable substrate in mammals. The body's reserves of carbohydrate, in the form of glucose and glycogen, are very limited and, except during the intervals immediately following meals, the body subsists primarily on calories supplied by stored fat. In contrast to its limited ability to store carbohydrate, the body has, perhaps unfortunately, an almost infinite capacity to expand its stores of fat. An understanding of the mechanisms by which the fat stores are laid down and by which they are mobilized is of central importance to an understanding of energy metabolism.

The sources of the adipose tissue triglycerides (TG) are several (Fig. 1). Triglycerides, carried in chylomicrons and also in low-density lipoproteins, can be incorporated into the depot fat (Bragdon & Gordon, 1958; Rodbell, 1960; Bezman, Felts & Havel, 1962; Gutman, Landau & Shafrir, 1962). Studies with doubly labeled triglycerides indicate that these are largely broken down to glycerol and free fatty acids, only the latter being efficiently retained in the depot triglycerides, although there is evidence that triglyceride can initially be taken up intact (Shapiro, 1961; Rodbell, 1960; Olivecrona, 1962a, b). Free fatty acids (FFA) can be taken up by adipose tissue both *in vitro* and *in vivo* but it does not appear that this is a major input form (Bragdon & Gordon, 1958). Glucose is probably the major precursor of adipose tissue triglycerides and the metabolism of glucose by adipose tissue has been extensively reviewed by Jeanrenaud (1961). As discussed below, glucose can give rise both to the glycerol moiety of the triglycerides, by way of α-glycerophosphate, and to the fatty acid moieties, by way of acetyl CoA. Finally, the amino acids can also contribute to the fat stores to the extent that they give rise to glucose or to fatty acids after deamination.

In 1956 Gordon & Cherkes at the National Heart Institute and Dole (1956) at the Rockefeller Institute independently made the important finding that the free fatty acids of the plasma constitute a major transport form. It now appears that mobilization of fatty acids from adipose

tissue occurs primarily and probably exclusively in the form of these free fatty acids (FFA)*. There is no evidence that net mobilization of depot triglycerides occurs as such. Thus, in contrast to the multiplicity of modes of input, we know of only one output form of any importance—the free fatty acids.

The final common path through which all traffic into the depot must funnel is the conversion of FFA and α-glycerophosphate to triglycerides. Before substrates can contribute to the triglyceride stores they must first be converted to these precursors. Traffic out of the depot depends upon hydrolysis of the stored glycerides to generate FFA. Thus, the key reactions that govern traffic into and out of the adipose tissue stores are triglyceride biosynthesis and degradation.

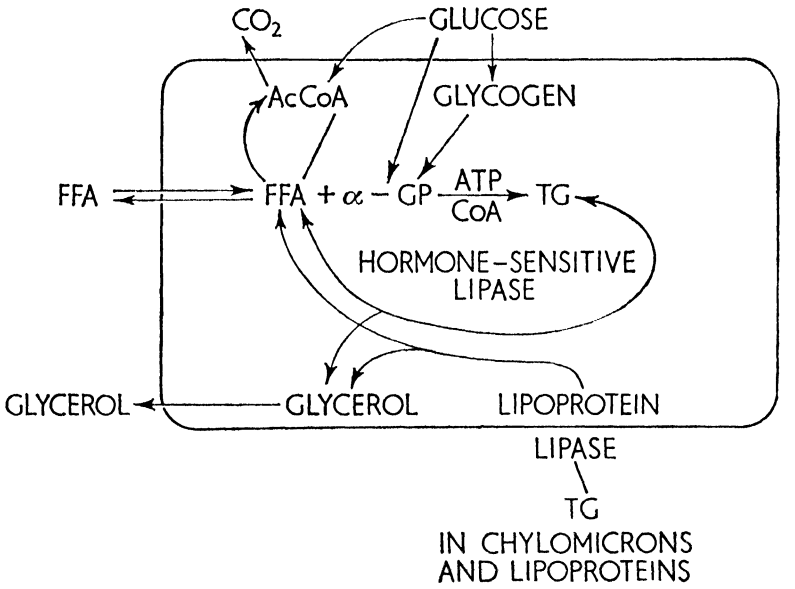


Fig. 1. Schematic representation of an adipose tissue cell indicating the major input and output pathways for fat deposition and mobilization.

The classical studies of Schoenheimer & Rittenberg (1936) established that depot fat is in a true dynamic state. Subsequent studies confirm that triglyceride synthesis and breakdown go on continuously even at steady state, although there is some question about what fraction of the triglyceride stores participate directly in this dynamic state (Hirsch, Farquhar, Ahrens, Petersen & Stoffel, 1960). If the rates of triglyceride synthesis and degradation are equal, the stores of depot fat will be

* The term *free fatty acids* (FFA) refers to long-chain fatty acids not covalently bonded. In the serum they are largely complexed with albumin. *Unesterified fatty acids* (UFA) and *nonesterified fatty acids* (NEFA) are alternative synonymous designations for this same fraction but the use of FFA is preferred (Bragdon, 1959). Other abbreviations used: ACTH = adrenocorticotropic hormone; TSH = thyroid-stimulating hormone; GH = growth hormone; DOCA = deoxycorticosterone acetate.

maintained at a constant level (Fig. 2). Net mobilization will occur if the steady state is disturbed by disproportionately increasing the rate of breakdown or decreasing the rate of synthesis. Conversely, net deposition will occur if the rate of synthesis is increased or the rate of breakdown is decreased. Obviously, the rates of both processes should be investigated when attempting to clarify the mechanisms by which metabolic or hormonal factors influence fat mobilization and deposition.

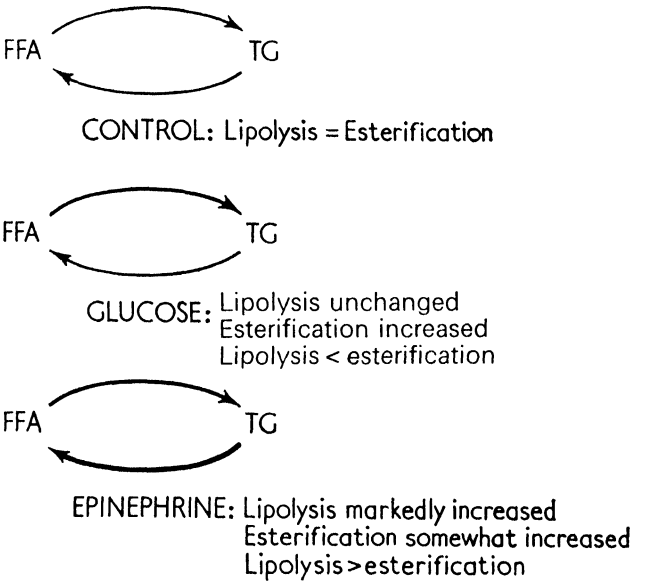


Fig. 2. The dynamic state of adipose tissue and the effects of glucose and of lipolytic hormones on it.

The purpose of the present paper is two-fold:

(1) To review recent *in vitro* studies on the mechanisms of synthesis and breakdown of triglycerides in adipose tissue cells and the manner in which hormones influence these processes.

(2) To attempt to evaluate and integrate a variety of biochemical and physiological studies relating to what we can call "the metabolic consequences of fatty acid mobilization".

TRIGLYCERIDE BIOSYNTHESIS AND DEGRADATION—MECHANISMS AND MEASUREMENTS

(a) *Triglyceride biosynthesis*

Synthesis of triglycerides in homogenates of rat adipose tissue has been shown to occur by way of the following sequence of reactions (Steinberg, Vaughan, Margolis & Karmen, 1960; Steinberg, Vaughan & Margolis, 1961; Rose & Shapiro, 1961):

(1) 3 fatty acid $\xrightarrow[\text{CoA}]{\text{ATP}}$ 3 fatty acyl CoA

(2) 2 fatty acyl CoA + α-glycerophosphate → phosphatidic acid

(3) phosphatidic acid → diglyceride + inorganic phosphate

(4) diglyceride + fatty acyl CoA → triglyceride.

The mechanism is seen to be the same as that for triglyceride synthesis in liver (Weiss & Kennedy, 1956; Tietz & Shapiro, 1956) and intestine (Dawson & Isselbacher, 1960). An important difference between the liver and adipose tissue, however, is the inability of the latter to utilize free glycerol in place of α-glycerophosphate. Whereas the liver contains an active glycerophosphokinase, adipose tissue lacks this enzyme (Wieland & Suyter, 1957; Margolis & Vaughan, 1962). Glycerol, then, is not utilized to any extent for triglyceride synthesis and it is also very poorly oxidized in adipose tissue (Shapiro, Chowers & Rose, 1957; Cahill, Leboeuf & Renold, 1960; Lynn, MacLeod & Brown, 1960; Steinberg, 1962). In a sense, glycerol can be regarded as an end-product of adipose tissue metabolism (derived from hydrolysis of triglycerides) and this, as discussed below, makes it possible to assess the rate of lipolysis *in vitro* directly by determining the rate of net accumulation of glycerol.

(b) *Triglyceride degradation*

As far as is known, net breakdown of triglycerides in adipose tissue occurs by simple lipolysis, i.e. there is no evidence for complex degradative mechanisms involving acceptors other than water.

When intact adipose tissue from fasting rats is incubated *in vitro* there is a net release of both FFA and glycerol into the medium. Even when there is net uptake of FFA into the tissue, as when the tissue is incubated in the presence of a high concentration of glucose, glycerol production continues (Leboeuf, Flinn & Cahill, 1959), indicating that lipolytic breakdown is continuing in the face of net triglyceride deposition. The absolute rate of glycerol production is not altered significantly by the presence of glucose in the medium (Vaughan, 1962a) but insulin does appear to reduce it under certain circumstances (Jungas & Ball, 1962). The hormones that stimulate FFA release *in vitro* simultaneously stimulate glycerol formation (Leboeuf *et al.*, 1959; Lynn *et al.*, 1960; Hagen, 1961; Vaughan & Steinberg, 1963a). It has been shown recently that these hormones activate a hormone-sensitive lipase system, as discussed below in greater detail.

(c) *Measurement of rates of triglyceride synthesis and breakdown in intact adipose tissue*

A number of investigators have taken the rate of incorporation of labeled FFA from the medium into tissue glycerides to be a measure of the rate of triglyceride synthesis (Shapiro, *et al.*, 1957; Bally, Cahill, Leboeuf & Renold, 1960; Steinberg, Vaughan & Margolis, 1960; Kerpel,

Shafrir & Shapiro, 1961; Dole, 1961; Shafrir & Kerpel, 1961). For reasons discussed in detail elsewhere (Vaughan, 1961a, b; Steinberg, 1962), these isotopic methods may, in adipose tissue, give results that are erroneous not only quantitatively but, under certain circumstances, even qualitatively.

An estimate of rates of triglyceride synthesis and of rates of lipolysis can be made using a nonisotopic "balance method" described elsewhere (Vaughan, 1962a, b; Vaughan & Steinberg, 1963a; Steinberg, 1962). One can estimate the total amount of fatty acid released from triglyceride stores during the course of an incubation by measuring the number of micromoles of glycerol produced (change in medium plus change in tissue content) and multiplying by three. At the same time the *actual* increment in FFA is measured (both in medium and in tissue). The latter value is always smaller, the difference representing the number of micromoles of FFA that were incorporated into glycerides during the incubation. To the extent that FFA and glycerol are metabolized via other pathways, the results obtained must be considered approximations but the errors are probably not large. A sample calculation to illustrate the use of the method is shown in Table 1.

Table 1. *Net balance method for calculating rates of lipolysis and esterification*

	Glycerol (μM/g)	*FFA* (μM/g)
Tissue at t_0	1·8	2·0
Tissue at 1 hour	0·5	0·8
Net change in tissue	−1·3	−1·2
Medium at t_0	0	0·2
Medium at 1 hour	2·6	1·5
Net change in medium	+2·6	+1·3
Overall change for system	+1·3*	+0·1

* This value $\times 3 (= +3{\cdot}9)$ is the μM of FFA released. The difference from +0·1 (=3·8 μM) represents the amount of FFA which must have been reesterified.

As shown in Fig. 3, the rates of lipolysis and esterification in rat adipose tissue freshly removed from normal fed rats were very nearly equal. The rates varied from 2·5 to 5 μEq of fatty acid per gram per hour. It should be stressed here that the rate of accumulation of glycerol or FFA in the medium need not—and sometimes definitely does not—reflect the true rates of their *net* production. Transfer from the tissue to the medium can continue even though there is no further net glycerol or FFA formation. Furthermore, the true rates of lipolysis and esterification do not necessarily remain constant even over the period of a one-hour incubation (Vaughan & Steinberg, 1963a). Particularly when quantitative comparisons are to be made these factors must be kept in mind and observations should be limited to periods during which the true rates involved can be demonstrated to be constant.

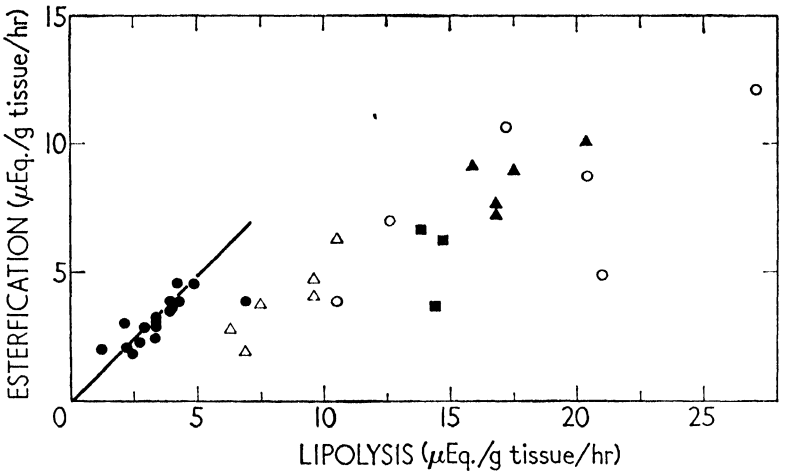


Fig. 3. Relationship of rate of esterification to rate of lipolysis in fat pads incubated for one hour in 3 ml Krebs bicarbonate medium containing bovine serum albumin, 30 mg/ml, one of each pair with, one without, hormone. ● = no hormone added, ■ = epinephrine, 0·1 μg/ml; ▲ = ACTH, 0·04 U/ml; ○ = TSH, 10 μg/ml; △ = GH, 200 μg/ml. Line drawn for rate of esterification = rate of lipolysis. (Vaughan & Steinberg, 1963b).

METABOLIC, HORMONAL AND NEURAL CONTROL OF FAT MOBILIZATION

(a) *Metabolic regulation—"glucose-cum-insulin"*

As discussed above, it appears that α-glycerophosphate is an obligatory acceptor scaffold onto which the fatty acids are stacked for storage. Since there is, even at steady state, a continuing minimum rate of lipolysis in adipose tissue, there must be a continuing minimum rate of supply of α-glycerophosphate, or precursors of it, to permit maintenance of the depot fat at steady state. White adipose tissue normally contains only very limited stores of glycogen and the pool of α-glycerophosphate itself is very small, less than 0·2 μmoles/g (Margolis & Vaughan, 1962). Under most circumstances, the α-glycerophosphate requirement must be met primarily by utilizing blood glucose. If this minimum requirement is not met and if lipolysis continues, FFA will be released at a rate in excess of that at which they can be reesterified. There will be net breakdown of triglyceride and release of FFA to the serum.

Adipose tissue taken from fasting animals and incubated *in vitro* shows a net release of FFA to the medium. Prefeeding the animal with glucose or adding glucose to the incubation medium prevents this release and can actually cause net uptake of FFA. Studies of net changes in FFA as well as studies on the rate of incorporation of labeled FFA show that glucose strongly stimulates incorporation of fatty acids into triglycerides (Raben & Hollenberg, 1960; Bally *et al.*, 1960; Steinberg *et al.*, 1960). On the other hand, addition of glucose alone or glucose plus insulin does not significantly alter the rate of glycerol production (Leboeuf *et al.*, 1959; Vaughan, 1962a). Thus glucose and insulin seem

uppress FFA release by accelerating esterification, without altering rate of lipolysis (Fig. 2). The effects of insulin on glucose metabolism l FFA release can be closely duplicated by simply raising glucose centrations to high levels (Jeanrenaud & Renold, 1959). Insulin ıe, in the absence of glucose, does *not* suppress FFA release (Engel & ite, 1960). It is reasonable, then, to attribute the primary effects of ılin on fat mobilization to its stimulation of glucose utilization by pose tissue. This is not to say that insulin is without other effects. ects on incorporation of acetate-^{14}C and pyruvate-^{14}C into protein e been observed in the absence of glucose (Krahl, 1959) and it has n reported that cytological changes compatible with the occurrence inocytosis can be seen in the absence of glucose (Barrnett & Ball, 0). What *can* be said is that the role of insulin in enhancing glucose ization would be sufficient to explain its effects on fat mobilization. lucose and insulin can probably be coupled both with regard to mate mechanism of action and with regard to physiological effect. viously the effects of insulin represent one form of hormonal control , as just pointed out, its effects with regard to fat mobilization are ely attributable to its enhancement of glucose utilization. It is con- ient to couple them and regard "glucose-cum-insulin" as the major conserving tandem in regulation of adipose tissue metabolism.

(b) *Direct hormonal stimulation of fat mobilization*

least 11 different hormonal factors have been shown to stimulate ase of free fatty acids *in vitro*, *in vivo*, or both:

(1) Epinephrine
(2) Norepinephrine
(3) Adrenocorticotropic hormone (ACTH)
(4) Glucagon
(5) Pituitary fraction H (Rudman) and Peptides I and II (Astwood)
(6) Chalmers factor (peptidic component isolated from urine of fasting subjects)
(7) Growth hormone
(8) Thyroid-stimulating hormone
(9) α- and β-intermedin
(10) Follicle-stimulating hormone
(11) Adrenal glucocorticoids

extensive literature on these hormones in relation to fat mobiliza- has been recently reviewed (Rudman, 1963) and no attempt will be le in this section to discuss the *in vivo* effects, some of which are ussed later (p. 124). Here we would like to review studies on the hanisms by which FFA release is stimulated *in vitro*.

. *Studies in intact tissues.* The first direct evidence that epinephrine eased the absolute rate of lipolysis in adipose tissue came from the ervation of Leboeuf *et al.* (1959) that the rate of glycerol production increased by incubation with the hormone. Their finding with

epinephrine has been amply confirmed and extended to many other hormones (Lynn *et al.*, 1960; Hagen, 1961; Vaughan, 1962b; Vaughan and Steinberg, 1962, 1963a,b). The magnitude of the effects obtained can be seen in Fig. 3, the values for lipolysis along the abscissa representing net glycerol formation (multiplied by 3 to express the results in terms of μEq FFA released from triglycerides). Results with norepinephrine (0·1 μg/ml) and glucagon (5 μg/ml), not shown in the Figure, were quantitatively similar to those obtained with epinephrine (0·1 μg/ml) (Vaughan and Steinberg, 1962, 1963b).

Studies of the effects of the lipolytic hormones on the rate of FFA esterification in the intact tissue have yielded conflicting results. Incorporation of labeled FFA from the medium is depressed by epinephrine (Steinberg *et al.*, 1960; Kerpel, Shafrir & Shapiro, 1960; Shafrir & Kerpel, 1960). The effect is not simply on the basis of dilution by unlabeled FFA released into the medium, since it can be demonstrated when the medium FFA specific radioactivity is kept constant by incubating in a large volume of medium of high FFA concentration (Steinberg, 1962). Making calculated corrections for changes in medium FFA specific radioactivity, Dole (1961) concluded that epinephrine had little effect on esterification. As mentioned above, p. 114 (C), the interpretation of studies of uptake of labeled FFA is beset by a number of uncertainties that make interpretation difficult.

The rate of incorporation of ^{14}C-glucose into triglyceride-glycerol is stimulated by epinephrine (Leboeuf *et al.*, 1959; Lynn *et al.*, 1960; Vaughan, 1961a, b). While this finding is compatible with an increased rate of triglyceride synthesis, it cannot be considered conclusive in the absence of direct information on the specific radioactivity of the immediate precursor, α-glycerophosphate.

Results obtained by the nonisotopic balance method described above (p. 115) provide straightforward evidence for an *increased* rate of esterification induced by several lipolytic hormones, including epinephrine ACTH and TSH (Vaughan & Steinberg, 1962, 1963b). This is shown in Fig. 3. While the calculated rate of esterification was markedly increased, the effects on lipolysis were greater, of course, leading to net FFA release. High concentrations (200 μg/ml) of growth hormone, on the other hand, caused significant increases in lipolysis without perceptibly affecting esterification in these experiments.

Most of the lipolytic hormones, then, appear to affect the steady state primarily by accelerating triglyceride breakdown. Triglyceride synthesis is accelerated concomitantly but to a lesser extent (Fig. 2).

2. *Studies in homogenates.* It has been shown that preincubation of adipose tissue with epinephrine or ACTH leads to activation of a tissue lipase or lipases, assayed in homogenates made subsequent to the incubation (Rizack, 1961; Hollenberg, Raben & Astwood, 1961; Hollenberg, 1962; Björntorp & Furman, 1962; Vaughan, 1962b). The magnitude of the effects obtained have been generally considerably smaller, in

percentage terms, than the effects of the hormones on rates of lipolysis in intact tissue. In part this may be attributable to the presence in adipose tissue of an enzyme very active in splitting monoglycerides (Berger, Vaughan & Steinberg, 1963). If Ediol, a commercial coconut oil emulsion, is used as substrate the monostearin in it (used as a stabilizer) is rapidly hydrolyzed by this lipase. This in effect adds a considerable "blank" value to the assay since, as shown below, the monoglyceride-splitting enzyme is *not* activated by hormone treatment. This rapid splitting of any monoglyceride contaminant in the substrate also makes the reaction rate nonlinear with time.

Another important factor in obtaining maximum hormone effects is preincubation of the tissue. The activity of the hormone-sensitive lipase decreases on incubation and only if the activity has been allowed to decline can a large difference between control and hormone-treated tissues be observed. In our own studies, the paired rat epididymal fat pads were preincubated in the absence of hormones for 80 minutes and hormone was added to one of the tissues for the last 10 minutes of the incubation. After homogenization in 0·15 M KCl, release of glycerol or FFA from breakdown of endogenous substrate in the whole homogenate was followed. As shown in Table 2, lipolytic activity was more than

Table 2. *Activating effect of hormones on lipase activity in preincubated adipose tissue**

	Lipolytic Activity (μmoles glycerol/g/30 min.)		
Hormone	*Control*	*Hormone-treated*	Δ
Epinephrine	1·0	2·6	+1·6 ± 0·2
ACTH	1·6	3·8	+2·2 ± 0·2
Glucagon	1·8	4·8	+3·0 ± 0·7
TSH	1·6	2·9	+1·3 ± 0·5

* Paired fat pads incubated 90 minutes. Hormones added to one of each pair during final 10 minutes of incubation. (Data of Vaughan, Berger and Steinberg.)

doubled under these conditions. The mean increase effected by ACTH after preincubation for 3 hours was 400% (Vaughan, 1962b; Vaughan & Steinberg, 1963b; Vaughan, Berger & Steinberg, unpublished data). It is important to note that addition of pure triglyceride emulsions to these homogenates did not increase overall lipolysis beyond that seen when only endogenous substrate was available. On the other hand, the hydrolysis of added monoglyceride was extremely active (Table 3) but

Table 3. *Failure of norepinephrine to stimulate "monoglyceridase" activity**

	Lipolytic Activity (μM glycerol/g/30m)		
Substrate	*Control*	*Treated*	Δ
Endogenous	0·9	2·7	+1·8 ± 0·26
Monostearin	50	55	+5 ± 3·5

* Five pairs of tissues incubated for 90 minutes. Norepinephrine added to one of each pair during last 10 minutes of the incubation. (Berger *et al.*, 1963.)

was essentially unaffected by prior hormone treatment. Recently it has been possible to prepare acetone-powder fractions that retain the ability to split monoglycerides but are inactive in splitting triglycerides (Berger *et al.*, 1963). Thus there are at least 3 different lipases in rat adipose tissue—lipoprotein lipase, a "monoglyceridase" and a hormone-sensitive lipase.

3. *Other metabolic effects of lipolytic hormones.* Vaughan (1960) has shown that epinephrine, norepinephrine, glucagon and ACTH can stimulate activation of phosphorylase in adipose tissue. The first three have been previously shown to have a similar effect in the liver (Sutherland & Rall, 1957) and ACTH has a similar action on the adrenal cortex (Haynes, 1958). In the latter tissues the mechanism of action has been shown to involve first the formation of cyclic 3′, 5′-adenosine monophosphate (3′,5′-AMP), which then stimulates conversion of inactive phosphorylase to active phosphorylase. Formation of 3′,5′-AMP has been demonstrated in adipose tissue (Vaughan, 1960) and epinephrine enhances its formation (Sutherland & Rall, 1960). It is tempting to speculate that 3′,5′-AMP is somehow involved, then, in activation of the lipase system. One finding difficult to reconcile with such a hypothesis is the fact that serotonin, which very effectively stimulates phosphorylase activation, has no effect on FFA release (Vaughan, 1960), nor does it interfere with epinephrine stimulation of FFA release when both are added at the same time. Still, in view of the parallelism between the actions of the other hormones on the two systems, it is difficult to believe that they represent completely independent effects. Further elucidation of the mechanism of lipase activation will undoubtedly suggest what the nature of the connection between the two may be.

Finally, mention must be made of the stimulation of glucose uptake effected by the catecholamines, ACTH, and glucagon (Cahill *et al.*, 1960; Lynn *et al.*, 1960; Vaughan, 1961b) which is associated with a greater relative stimulation of the rate of oxidation by way of the pentose phosphate pathway. Cahill and co-workers suggest that the rise in intracellular FFA concentrations may induce the increase in glucose utilization but Vaughan has shown that at lower concentrations of hormone it is possible to retain near-maximal effects on FFA release with little or no effect on glucose uptake. Whatever the mechanism may be it is doubtful that the effect is necessary for the fat-mobilizing action of the hormones. Actually, glucose uptake might be expected to damp the effects on net FFA release.

Adipose tissue seems to be most undiscriminating and unselective in its response to hormones. The liver phosphorylase system responds to catecholamines and glucagon but not to ACTH, the adrenal cortex responds to ACTH but not to catecholamines or glucagon. Adipose tissue phosphorylase is activated by all three. The list of structures that can stimulate FFA release is almost embarrassingly long. This peculiarly undifferentiated state with regard to hormonal effects is

intriguing from a developmental point of view. Since the hormonal response is so easily measured, study of adipose tissue may be quite valuable in ultimately elucidating hormone-enzyme relationships.

(c) *Indirect Hormonal Regulation—"permissive" effects of adrenocortical steroids and thyroid hormones*

The effectiveness of epinephrine in mobilizing FFA has been shown to be markedly impaired in adrenalectomized rats (Shafrir, Sussman & Steinberg, 1959b, 1960) and dogs (Shafrir & Steinberg, 1960). Epinephrine responsiveness could be restored by pretreating the animals, previously maintained on salt or deoxycorticosterone alone, with cortisone. The latter was used in doses that did not by themselves significantly alter FFA levels. Similar results were obtained in hypophysectomized animals (Figs. 4 and 5). It has been shown further that adipose tissue taken from adrenalectomized rats given an injection of epinephrine shortly before sacrifice releases FFA at a lower rate than tissue taken from normal animals similarly pretreated with epinephrine. Finally, the response of fat pads to epinephrine added *in vitro* is much smaller in tissues taken from adrenalectomized rats (Shafrir *et al.*, 1960; Reshef & Shapiro, 1960) but the response of the tissues is normal when the adrenalectomized rats have been maintained on cortisone.

While adrenocortical hormones can, at high concentrations, have a direct effect on FFA release (Jeanrenaud & Renold, 1960) and can under special circumstances have marked effects on fat mobilization (Scow and Chernick, 1960), it is to be stressed that in the studies under discussion, cortisone alone in the doses given did *not* itself significantly alter plasma FFA levels. The effect must be regarded not as a synergistic one in the usual sense, but rather as a "permissive" effect—the glucocorticoid hormone in some unknown way conditions the metabolic stance of the adipose tissue to permit maximum response to the catecholamine.

A similar permissive action can probably be ascribed to thyroid hormone. Rich, Bierman & Schwartz (1959) first called attention to the high plasma FFA levels of hyperthyroid patients and showed that treatment of normal subjects with triiodothyronine led to progressive rises in FFA levels. At about the same time Goodman & Knobil (1959b) noted that hypophysectomized monkeys show a damped FFA response to injected epinephrine. Their responsiveness was restored by pretreatment with TSH or triiodothyronine. It should be noted that cortisol and ACTH were ineffective in restoring epinephrine responsiveness in these experiments.

Debons & Schwartz (1961) found that adipose tissue from rats treated with triiodothyronine released FFA at a higher than normal rate and showed an exaggerated response to epinephrine *in vitro*. Tissue from rats treated with propylthiouracil released FFA more slowly and did

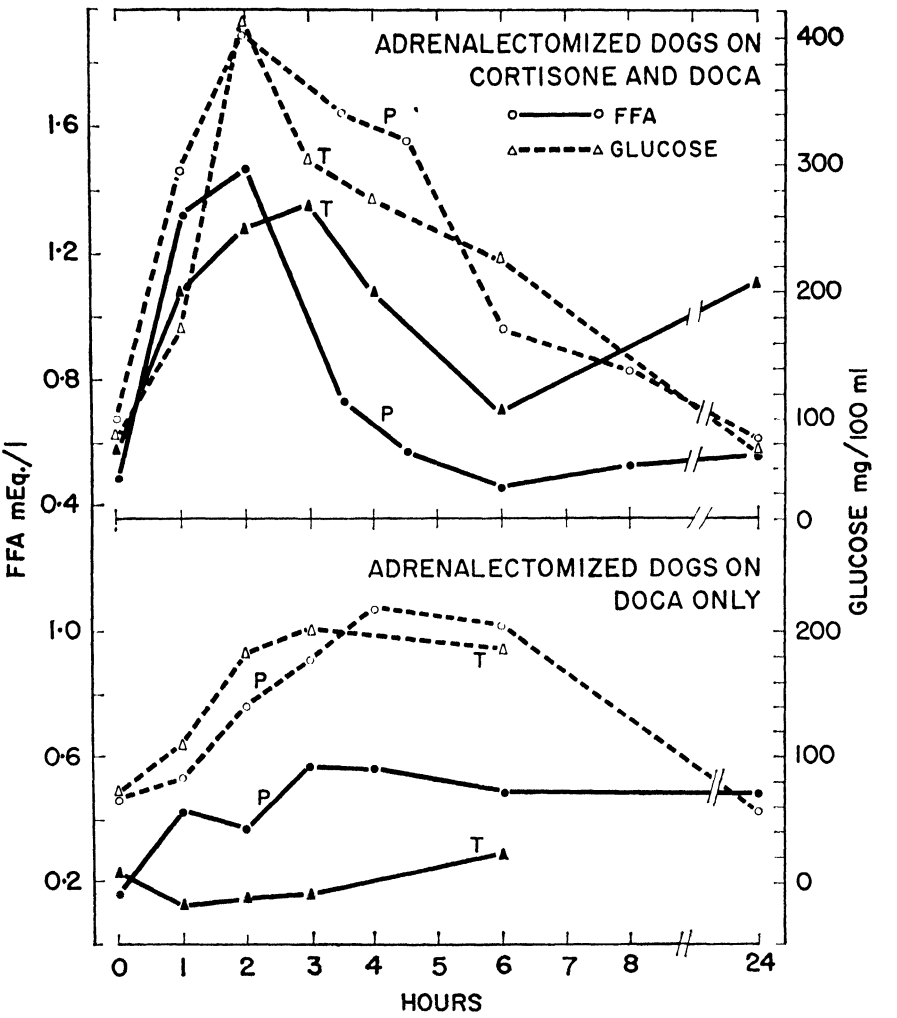


Fig. 4. Effects of subcutaneous epinephrine in oil (0·6 mg/kg) on FFA and glucose levels in adrenalectomized dogs. *Lower frame*: adrenalectomized dogs given only DOCA. *Upper frame*: same adrenalectomized dogs treated with cortisone before epinephrine injection. (Shafrir & Steinberg, 1960.)

not respond to epinephrine. These findings have been confirmed and extended by Deykin & Vaughan (1963) who have, by measuring glycerol formation, shown that both lipolysis and esterification are increased in tissues of hormone-treated rats.

Addition of triiodothyronine *in vitro* to normal adipose tissue does not affect FFA release. The animals must be pretreated. It is not yet established, then, whether the accelerated lipolysis that follows treatment with thyroid hormone reflects a direct action (but one that is slow to develop) or whether the effect is due exclusively to potentiation of the action of endogenous catecholamines. The potentiation of catecholamine

activity is clear and, whether *strictly* permissive or not, the effect of thyroid hormone probably represents another type of *indirect* or *conditional* hormone regulation.

(d) *Neural and neurohumoral control*

It has been recognized for some time that the direct innervation of adipose tissue plays an important role in facilitating fat mobilization

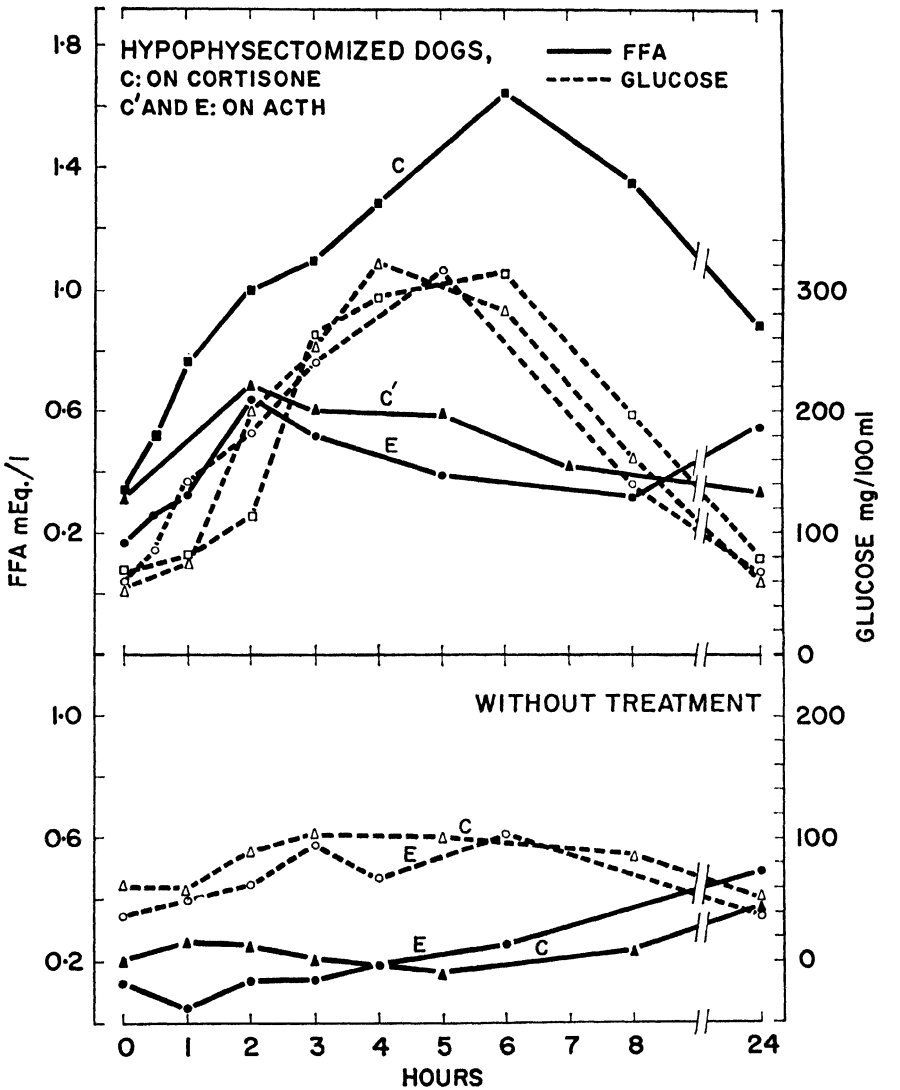


Fig. 5. Effects of subcutaneous epinephrine in oil (0·6 mg/kg) on FFA and glucose levels in hypophysectomized dogs. *Lower frame*: hypophysectomized dogs given no treatment. *Upper frame*: same hypophysectomized dogs given cortisone or ACTH. (Shafrir & Steinberg, 1960.)

(Wertheimer & Shapiro, 1948 (review); Levy & Ramey, 1960). Recent studies relate this function to FFA release. Thus Correll (1961) has demonstrated that electrical stimulation of the nerve supply to the isolated rat fat pad *in vitro* can double the rate of output of FFA. Havel & Goldfien (1959) showed that ganglionic blockade with hexamethonium in dogs reduces plasma FFA levels. Measurements of turnover established that the effect was due to suppression of FFA release. Since norepinephrine is the chemical mediator at postganglionic sympathetic nerve endings (Euler, 1956) it is likely that the biochemical mechanisms involved are like those due to administration of exogenous catecholamines. How important tonic nerve impulses may be in determining basal rates of FFA release is not known. However, it has been shown that the sympathetic discharge associated with emotional stress can markedly elevate plasma FFA levels in man (Cardon & Gordon, 1959; Bogdonoff, Estes & Trout, 1959). Here the extent to which adrenomedullary activity and direct peripheral sympathetic discharge, respectively, contribute to the observed responses has not been established. As discussed below, these findings indicate an important psychic-somatic interaction that could explain changes not only in blood FFA levels but also in levels of other blood lipids induced by emotional or physical stress (see p. 128 (b)).

METABOLIC CONSEQUENCES OF FFA MOBILIZATION

(a) *Deposition of fat in the liver*

The fact that development of certain forms of fatty liver in experimental animals depends in part on mobilization of fat from the depots was recognized years ago. Now that we know in what form the depot fat is mobilized it becomes possible to evaluate the importance of this factor more directly. To determine whether excessively rapid mobilization of FFA could in itself produce a fatty liver (in the absence of any prior liver damage), anesthetized dogs were given constant intravenous infusions of norepinephrine for 8 hours (Feigelson, Pfaff, Karmen & Steinberg, 1960, 1961). This raised serum FFA levels to about 2 μEq/ml and maintained them at this high level over the 8-hour period. Serial biopsies taken from an animal treated this way showed a progressive rise in liver glyceride content, reaching values about 5-fold greater than control values (Fig. 6). Data from 7 such experiments are summarized in Table 4.

We had to consider the possibility that the catecholamine might be exerting a hepatotoxic action. It has been shown that blood norepinephrine is largely inactivated by a single passage through the liver. Hence one might expect that by perfusing via the portal vein it would be possible to expose the liver to the highest possible concentrations of infused norepinephrine and yet not raise serum FFA levels markedly. As shown in Fig. 7 this expectation was borne out. That the responsive-

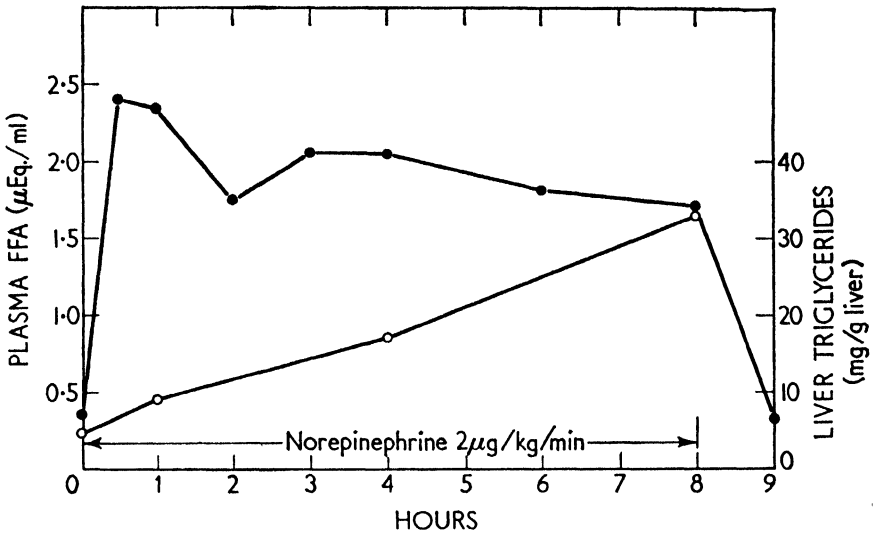


Fig. 6. Changes in plasma FFA level (●———●) and liver triglyceride concentration (○———○) during continuous femoral vein infusion of norepinephrine. (Feigelson *et al.*, 1961.)

Table 4. *Increase in liver glyceride concentration induced by infusing norepinephrine for eight hours**

	Liver glycerides (mg/g)		
	Zero time	*8 hours*	*Percentage change*
Saline infusion (4)	5·1	7·1	+39%
Norepinephrine via femoral vein (7)	6·3	36·9	+490%
Norepinephrine via portal vein (4)	10·8	13·7	+27%

* Anesthetized dogs given 1 to 2 μg/kg/min. (Feigelson *et al.*, 1961.)

ness of the animal's adipose tissue was not impaired was clearly shown by the immediate sharp rise in serum FFA when the infusion was switched from the portal vein to a peripheral vein. As shown in Table 4, intraportal infusions, at the same dosage rate used for peripheral infusions, did not lead to fatty liver. On the basis of these results it was proposed (Feigelson *et al.*, 1961) that elevation of serum FFA concentrations *per se* was an adequate basis for development of fatty liver. As schematically indicated in Fig. 8, an excessively rapid mobilization of FFA to the liver, even though the latter is functioning normally, can exceed the capacity of the liver to dispose of fatty acids by oxidation or resecretion in ester form via the lipoproteins. It was further proposed that the development of fatty livers after administration of hepatotoxic agents might occur even with a normal rate of delivery of FFA to the liver, since the damaged liver would have a lower than normal capacity to dispose of the FFA taken up. In short, fatty liver would be expected

to occur on the basis of an imbalance between the rate of uptake of FFA by the liver and the capacity of the liver to dispose of those FFA: (a) by oxidation; (b) by esterification and retransport in lipoprotein form. In many cases it is obviously quite possible that both of these basic mechanisms are simultaneously operative.

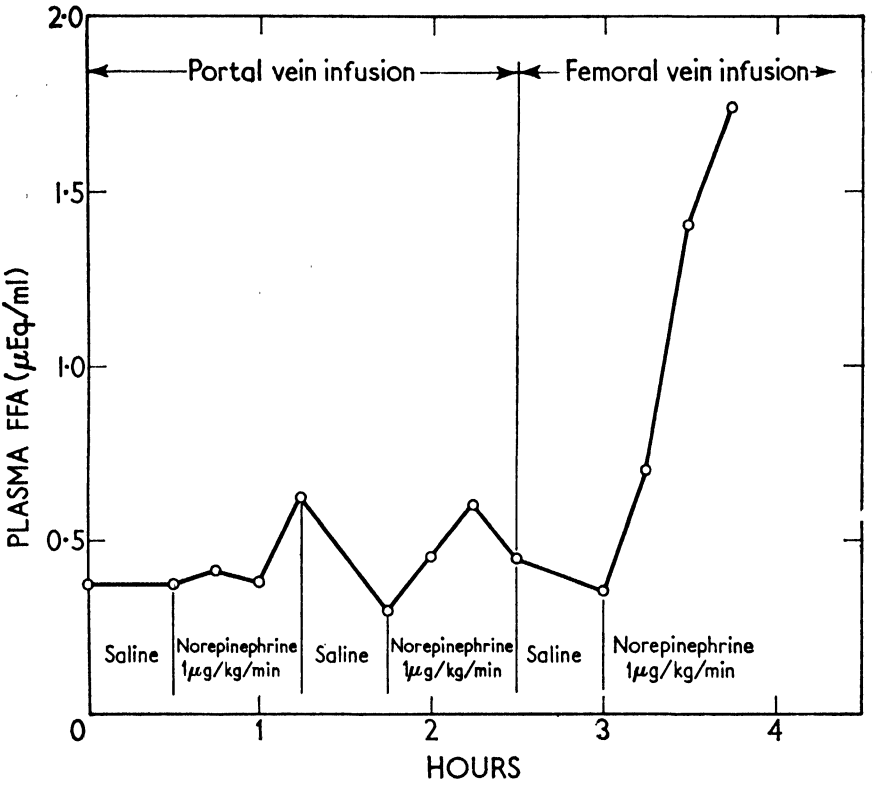


Fig. 7. Plasma FFA levels during intraportal infusion of norepinephrine contrasted with FFA levels during intrafemoral infusion at the same dose rate. (Feigelson *et al.*, 1961.)

Fat deposition in perfused liver induced by elevated FFA concentrations. Recently we have obtained still more direct evidence implicating serum FFA levels as an adequate basis for inducing fat deposition in the liver. Rat livers were perfused with oxygenated red blood cell-albumin-saline mixtures to which FFA were added at different concentrations. It was found that when the initial FFA concentration of the perfusing fluid was very high (2–3 μEq/ml) the rate of uptake by the perfused liver was extremely rapid and that most of the FFA taken up could be accounted for in the liver triglyceride fraction (Nestel & Steinberg, 1963). As shown in Table 5, in 90 minutes as much as 126 μEq of perfusate FFA was taken up and the total liver triglyceride fatty acid content rose from 23 μEq/g to over 33 μEq/g, an increase of almost 50% in 90 minutes. When the uptake of FFA was followed in shorter

perfusions the fractional removal in a single passage of perfusate through the liver could be calculated to be 27%, in close agreement with the fractional uptake calculated by Fine & Williams (1960) from *in vivo* dog studies.

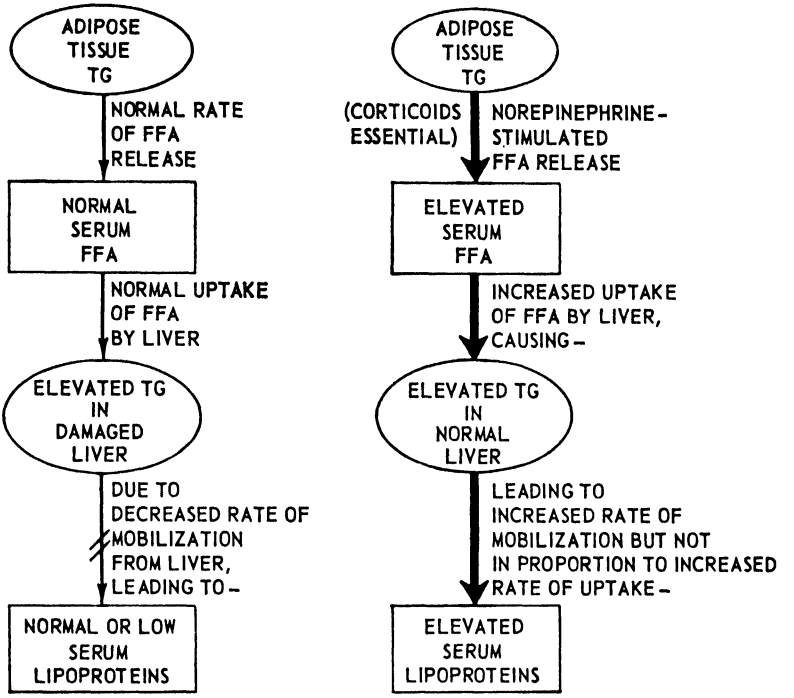


Fig. 8. Proposed scheme summarizing the role of plasma FFA in the development of fatty liver in a normal liver (right) and in a damaged or metabolically altered liver (left). Mobilization, as used in this scheme, includes oxidation to CO_2 or secretion into the plasma as component lipids of the lipoproteins, or both. (Feigelson *et al.*, 1961.)

Table 5. *Net changes in liver and in perfusate glyceride content as a function of perfusate FFA concentration**

Fatty Acid Added to Perfusate	Total FFA Concentration of Perfusate (μEq/ml) Initial	Final	Total FFA Uptake (μEq)	Increase in Liver Glyceride Fatty Acids (μEq)	Increase in Perfusate Glyceride Fatty Acids (μEq)
None (2)	0·36	0·20	4	—	1·5
Linoleate (2)	2·10	0·28	55	40	3·5
Palmitate (2)	2·35	0·27	62	50	3
Linoleate + Palmitate (4)	3·97	0·40	126	98	7·5

* Isolated rat livers perfused for 90 minutes. The livers perfused weighed 7·10 to 9·28 g. The zero time glyceride fatty acid content of liver determined in matched controls averaged 23 μEq per gram or about 170 μEq per liver. The zero time glyceride content of the *total* pool of perfusing fluid was 18 μEq of glyceride fatty acid. (Nestel and Steinberg, 1963.)

Role of the adrenal in the development of fatty livers. We have discussed above the essential role of the adrenal cortex in facilitating catecholamine-induced FFA mobilization (Shafrir *et al.*, 1959b, 1960; Shafrir & Steinberg, 1960; Reshef & Shapiro, 1960). Considering those findings together with the present findings, we can offer a rational, although not necessarily complete, explanation for the repeatedly confirmed observation that adrenalectomy protects against development of certain types of fatty liver. Phosphorus poisoning, exposure to cold, prolonged fasting or treatment with epinephrine will produce fatty livers in rats, but not if the adrenal glands have been removed (Verzar & Laszt, 1936; MacKay, 1937; LeBlond, Thoai, Nguyen-van & Segal, 1939; Wool, Goldstein, Ramey & Levine, 1954). Pretreatment of the adrenalectomized animals with crude adrenocortical extracts or corticosteroids restored their susceptibility to development of fatty livers. There is evidence that the adrenal medulla, removed also in these studies, also plays a role (Wool, *et al.*, 1954). However, the fact that cortical extracts or corticosteroids alone were sufficient to restore susceptibility in most studies suggests that the role of medullary secretion is not of overriding importance.

Steinberg & Shafrir (1960) proposed, then, that in the kinds of studies cited above the development of the fatty liver depends upon reaching some threshold rate of delivery of FFA from the adipose tissue to the liver. The threshold will depend on the extent to which the capacity of the liver to metabolize FFA has been compromised. Since intact adreno-medullary function is a condition for maximal rates of FFA mobilization, at least in response to catecholamine stimulation, adrenalectomy will tend to protect the liver from loads of FFA it is unprepared to handle (Fig. 8).

As discussed above, whether or not fat accumulates in the liver will depend on the *balance* between rate of uptake and rate of mobilization and oxidation. Merely because adrenalectomy or administration of drugs that block FFA mobilization prevents fatty liver due to a given agent, it does not necessarily follow that the causative agent *stimulates* FFA mobilization. The effect of the agent may still be exclusively hepatic. Protection against such an agent could still be afforded by reducing the rate of FFA mobilization to *subnormal* levels. Whether adrenal function is also important in other respects (e.g. by influencing uptake of chylomicron and lipoprotein triglycerides in the liver or by influencing fatty acid oxidation in the liver) has not been thoroughly explored. The fact that adrenalectomy protects against the fatty liver induced by ethionine (Wool & Goldstein, 1953) whereas, according to Olivecrona (1962a), FFA uptake is normal in ethionine-treated rats, suggests in fact some additional role for the adrenal.

(b) *Elevation of serum lipoprotein levels*

When large doses of epinephrine are given subcutaneously in oil suspension, so as to maintain hormonal activity over a long period of time,

it is possible to produce consistent, unequivocal elevations of serum lipoproteins in dogs, rats and rabbits (Kaplan, Jacques & Gant, 1957; Shafrir, Sussman & Steinberg, 1959a, b, 1960; Dury, 1957). However—and this in part explains earlier negative results—the elevation is a delayed response. The serum FFA levels begin to rise almost immediately and reach a peak value at about 2 hours. By contrast the cholesterol, phospholipid, and triglyceride levels remain virtually unchanged for 6 to 12 hours. By 24 hours very distinct changes are seen. The responses of fasted dogs to a single dose of epinephrine are illustrated in the first line of Table 6. If daily injections are continued the serum cholesterol level can be increased to more than twice control levels.

Table 6. *Serum lipid changes induced in dogs by daily injection of epinephrine**

Days of Epinephrine Treatment	No. of Dogs in group	Percentage Increase Above Control Levels		
		Total Cholesterol	Phospholipids	Triglycerides
1	19	28·6 ± 7·9	41·0 ± 13·9	24·8 ± 34·4
3	7	60·1 ± 25·8	48·2 ± 23·6	33·2 ± 13·3
6–8	6	90·9 ± 50·0	52·7 ± 22·5	16·9 ± 49·0

* Subcutaneous injections of epinephrine in oil (1 mg/kg). Blood samples were taken after an overnight fast. (Shafrir, Sussman & Steinberg, 1959 or 'a' or 'b'.)

In rats the changes in plasma lipids induced by epinephrine in oil were very similar to those seen in dogs (Shafrir *et al.*, 1960). Cholesterol and phospholipid levels rose but triglyceride levels were not changed. In striking contrast, the rabbit responds to depot epinephrine *predominantly* with a dramatic three-fold rise in plasma triglyceride concentrations at 24 hours (Dury, 1957). Cholesterol levels rose by almost 50% and phospholipid levels by about 30%.

Rudman, Seidman, Brown & Hirsch (1962) have carefully studied the time course of the changes in plasma lipid levels in rabbits after subcutaneous injection of purified porcine fraction H, a polypeptide isolated from the pituitary gland. Fraction H is very potent in stimulating release of FFA from rabbit adipose tissue (Rudman, Hirsch, Kendall, Seidman & Brown, 1962). Again, as after epinephrine, the immediate response was a rise in plasma FFA levels which reached maximal levels within 2 hours and remained high for at least 8 hours. By contrast, the serum triglyceride levels were unchanged at 2 and even at 4 hours after injection but rose to very high values at 8 hours. Cholesterol and phospholipid levels rose also but not as strikingly. Similar results have been obtained by Friesen, Barrett & Astwood (1962) with highly potent pituitary peptides, probably closely related to Fraction H.

In all of the studies mentioned above, the elevation of lipoprotein

levels was delayed and, where it was studied, the elevation of FFA levels was found to be the earlier response. Is the mobilization of FFA the *cause* of the subsequent rise in lipoprotein levels or is the latter an independent and possibly unrelated phenomenon? We undertook to study this from the point of view that epinephrine might exert its effects by stimulating pituitary and adrenal function. It was well-established by *in vitro* studies that epinephrine could act *directly* on adipose tissue to stimulate FFA mobilization and we anticipated that adrenalectomy in dogs might block the lipoprotein response to epinephrine without altering the FFA response. Unexpectedly both responses were reduced or abolished in adrenalectomized animals given only salt or DOCA (Shafrir *et al.*, 1959a, b; Shafrir & Steinberg, 1960) (Fig. 4 and Table 7). This dependence of epinephrine-stimulated FFA mobilization on adrenal function has been confirmed in several studies

Table 7. *Effect of adrenalectomy on changes in serum lipids induced by epinephrine**

	Percentage change		
	TC	*PL*	*TG*
Intact dogs	+17·7	+32·3	+1·3
Adrenalectomized dogs on DOCA alone	+0·3	+1·1	−2·3
Adrenalectomized dogs on DOCA plus cortisone**	+18·1	+15·1	−24·1

* Dogs fasted overnight before injections (0·6 mg/kg subcutaneously) and for the following 24 hours. Three animals in each group.
** Cortisone (3 to 5 mg/kg) given for 7 to 10 days before testing responsiveness to epinephrine. (Shafrir & Steinberg, 1960.)

(Reshef & Shapiro, 1960, rats; Havel & Goldfien, 1959, dogs; Shafrir *et al.*, 1960, rats). By giving cortisone for 7–10 days just before testing with epinephrine it was possible to restore the responsiveness. Both the rise in plasma FFA (Fig. 4) and the rise in plasma cholesterol and phospholipids (Table 7) were now comparable to those seen in intact dogs. These results strongly suggested that excessively rapid FFA mobilization was indeed the cause of the subsequent rise in plasma lipoprotein levels. The possibility remained, however, that the two responses were due to separate modes of action of epinephrine at different sites but that *both* were dependent somehow on adrenocortical function.

Recently, direct evidence has been obtained for an effect of FFA level on lipoprotein production by the liver (Gidez, Roheim & Eder, 1962; Nestel & Steinberg, 1963). Rat livers were perfused with red cell-albumin-saline mixtures containing different levels of FFA and the amounts of lipoprotein or lipoprotein triglyceride secreted into the perfusate were determined. As shown in Table 5, the increment in perfusate triglycerides observed when the initial FFA concentration was high was several times as great as the increment when the initial FFA concentration was low. Furthermore, the fatty acid composition of the perfusate triglycerides was distorted in a manner indicating

utilization of the fatty acid added to raise the initial FFA level of the perfusate. That is, when palmitate alone was added to the initial perfusing fluid at high concentration, the newly secreted triglycerides added to the perfusate were enriched in palmitic acid. These results provide strong evidence for an unmediated effect of FFA concentration on rate of lipoprotein secretion. By what mechanism this occurs we shall probably not be able to say until the more fundamental properties of the lipoprotein-synthesizing and lipoprotein-secreting mechanisms become known.

The kinetic studies of Havel and co-workers (Havel, Felts & Van Duyne, 1962, in rabbits; Havel, 1961, in man) are highly relevant to the present argument. These studies, based on measurement of the fate of injected labeled FFA, show quite conclusively that the major source for the triglyceride fatty acids of plasma is, in fact, the FFA of plasma. In other words, the FFA taken up by the liver are incorporated into triglycerides and resecreted, mostly in very low density lipoproteins, without much dilution by other fatty acids.

In summary, there are now several lines of evidence to support the conclusion *that elevated plasma levels of FFA, and the consequent elevated rate of uptake of FFA by the liver, lead in a rather direct manner to an increase in the rate of production and secretion of lipoproteins into the plasma.* As shown in Fig. 9, there is a physiological fatty acid cycle

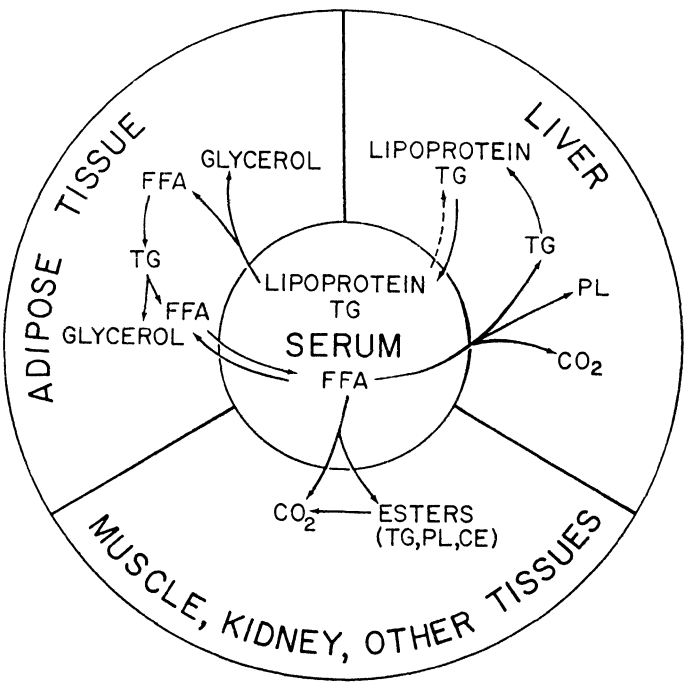


Fig. 9. Schematic representation of FFA metabolism emphasizing the "fatty acid cycle": adipose tissue triglycerides → adipose tissue FFA → serum FFA → liver triglyceride → lipoprotein triglyceride → adipose tissue FFA and triglyceride.

that reads: adipose tissue triglycerides–serum FFA liver triglycerides–lipoprotein triglycerides in liver–lipoprotein triglycerides in serum–adipose tissue triglycerides.

There are a number of physiological and pathological conditions in which elevated plasma lipid levels may be attributable wholly or in part to such a mechanism. The marked plasma lipid elevations in the poorly treated *diabetic* (and the fatty changes in the diabetic liver) may be secondary to the high plasma FFA levels seen in this condition. When the diabetic is treated with insulin the plasma FFA levels, which are decidedly elevated, return very promptly toward normal (Bierman, Schwartz & Dole, 1957). This occurs even before glucose levels return to normal and well before the hyperlipoproteinemia is corrected. In other words, the time course is compatible with the proposition that the initial effect of insulin therapy is to suppress FFA release by improving glucose utilization in adipose tissue. This can occur without large enough increases in total body glucose utilization to lower blood glucose levels. Only as glucose utilization in all of the peripheral tissues is enhanced by continuing treatment do blood glucose levels fall. Correcting the high FFA levels and relieving the liver of the excessive load of fat coming from the periphery ultimately reduces hepatic lipoprotein production and returns plasma lipoprotein levels toward normal.

Prolonged fasting increases plasma FFA levels and the moderate hyperlipemia seen in *fasting* individuals may be another example in the category under discussion.

Of particular interest to us is the elevation of plasma lipids in man under conditions of emotional stress (Wertlake, Wilcox, Haley & Peterson, 1958). It has been shown that fear or anger can acutely raise plasma FFA concentrations to high levels (Cardon & Gordon, 1959; Bogdonoff *et al.*, 1959) presumably secondary to overactivity of the sympathetic nervous system. Emotional stress is known to be associated with increased rates of adrenomedullary and adrenocortical secretion, both of which appear to be essential for achieving maximum rises in serum cholesterol and phospholipid levels in our dog studies. We have proposed that the hyperlipidemia of stress may have a straightforward neurohumoral basis, then, triggered by cerebral and hypothalamic stimuli but ultimately bringing into play the mechanisms discussed above (Shafrir & Steinberg, 1960).

(c) *Increase in rate of FFA oxidation*

In vitro studies have established that the rate of conversion of palmitate-1-^{14}C to $^{14}CO_2$ by skeletal muscle, diaphragm and liver is roughly proportional to the concentrations of FFA in the medium and depends on the ratio of FFA to albumin in the medium (Fritz, Davis, Holtrop & Dundee, 1958; Eaton & Steinberg, 1961; Shtacher & Shafrir, 1963; Rose, Vaughan & Steinberg, unpublished results). The same effect has

been demonstrated in perfused rat liver (Nestel & Steinberg, 1963) (Table 8).

Table 8. *Fate of palmitate-1-^{14}C during 30-minute perfusion of rat liver at low and at high initial FFA concentrations*

Initial FFA concn. (μEq/ml)	A-V diff. in O_2 sat'n. (Δ%)	Calculated minimum amount of palmitate (μEq) converted to*:				
		CO_2	Liver glycerides	Liver PL	Perfusate glycerides	Perfusate PL
0·36	14	0·04	0·75	0·68	0·016	0·004
2·06	26	0·74	26·1	4·6	0·76	0·008

* Observed total radioactivity in fraction (c.p.m.) divided by specific radioactivity of palmitate in perfusing fluid (c.p.m./μEq). (Data of Nestel and Steinberg, 1963.)

These studies in isolated tissues, however, leave open the question of whether in the intact animal there are hormonal and metabolic factors that modulate the apparent direct effect of FFA concentration on FFA oxidation. While oxidation by muscle of labeled FFA in the medium is increased, no concomitant increase in total oxygen consumption can be detected (Eaton & Steinberg, unpublished observations). This may reflect substitution of exogenous for endogenous caloric substrate. Evidence that this occurs in liver slices has been obtained, in that the respiratory quotient shows a distinct fall even though total oxygen consumption does not change (Eaton & Steinberg, unpublished observations).

Recent studies in normal human volunteers (Steinberg, Nestel, Buskirk & Thompson, 1963; Steinberg, Eaton, Buskirk & Thompson, unpublished data) showed that the rate of conversion of plasma palmitate-1-^{14}C to respiratory $^{14}CO_2$ could be markedly enhanced when the plasma FFA level was raised by infusing norepinephrine. A number of investigators have noted that the rate of conversion of injected labeled FFA to CO_2 in experimental animals is decreased by glucose-feeding and enhanced by fasting. Rates of FFA mobilization and plasma FFA levels are low in the former and high in the latter.

From these *in vitro* and *in vivo* studies it seems that plasma FFA concentration is almost certainly a factor controlling rates of FFA oxidation, just as glucose level is a factor determining its own rate of utilization (Soskin & Levine, 1937). In the latter case, however, we know that availability of insulin is, under normal conditions, a modulating factor of greatest importance. It is not yet known whether there are similar important hormonal effects on uptake and utilization of plasma FFA. The relative importance of substrate concentration in plasma *in vivo* as a determining factor can only be evaluated with further physiological and biochemical studies.

(d) *Production of ketone bodies*

Starvation and diabetes mellitus, conditions associated with ketosis, are also conditions in which plasma FFA levels tend to be high. The classical studies of Houssay & Biasotti (1931) and Long & Lukens (1936) established that hypophysectomy or adrenalectomy tends to prevent diabetic ketosis. Recently Scow & Chernick (1960) have presented evidence that in the rat it is primarily through secretion of ACTH that the pituitary influences ketogenesis, by stimulating production of glucocorticoids by the adrenal cortex. They have further shown that the rate of ketogenesis is a function of the amount of fat stored in the liver. Since lipogenesis is impaired in the diabetic state, the excess liver fat probably reflects transport of FFA from adipose tissue to the liver. If this is the case, the protection afforded by hypophysectomy or by adrenalectomy could be secondary to the impaired FFA mobilization associated with these procedures, as we have suggested previously (Shafrir & Steinberg, 1960). The extent to which the adrenal steroids influence FFA mobilization by a direct action (Scow & Chernick, 1960; Fain, Scow & Chernick, 1963) and the extent to which they do so by potentiating the action of catecholamines or other lipolytic hormones is not yet clear. In either case it seems very likely that the end result hinges on rates of mobilization of FFA, which are then converted to ketone bodies in the liver either directly (Gordon & Fredrickson, 1958) or after prior incorporation into liver triglycerides (Scow & Chernick, 1960).

(e) *Calorigenic effect*

The rise in total body oxygen consumption following administration of epinephrine has been extensively investigated but the mechanism of this calorigenic effect remains uncertain (Griffith, 1951; Ellis, 1956). Eaton & Steinberg (1961) proposed that the effect might be in part secondary to the FFA mobilization induced by the catecholamine, since FFA utilization by isolated tissues appears to be a function of FFA concentration. Further evidence along these lines was discussed above in (c) p. 132.

Recently we have shown that norepinephrine has a clearly demonstrable calorigenic effect in normal human subjects (Steinberg *et al.*, 1963) and that this correlates with an increase in FFA turnover in the plasma (Table 9). Since norepinephrine has only weak glycogenolytic effects, the interpretation of these studies is less complicated than in the case of epinephrine administration. Changes in blood glucose and lactic acid were minimal. Whether the effect was directly attributable to the high FFA levels *per se* or to other concomitant but independent effects of norepinephrine could still not be decided.

The results shown in Table 10 further support the direct relationship of fat-mobilizing action to calorigenic action. Pronethalol, a blocking agent of adrenergic β-acceptors (Black & Stephenson, 1962; Dornhorst

& Robinson, 1962) has been shown to block the FFA response to epinephrine in man (Pilkington, Lowe, Robinson & Titterington, 1962). After preliminary studies had established that the FFA response to

Table 9. *Effects of norepinephrine infusions in normal young adults**

Control level of FFA in plasma	0·58 ± 0·19 μEq/ml
Level of FFA in plasma during norepinephrine infusion	1·28 ± 0·27 μEq/ml
Percentage increase in turnover rate of plasma FFA**	74 ± 53·2%
Percentage increase in oxygen consumption	21 ± 8·5%

* Eleven subjects, ages 17 to 25, given continuous intravenous infusion of norepinephrine at rates of 10 to 21 μg/min. for periods ranging from 10 to 26 minutes. Values shown were determined at the time of the peak rise in oxygen consumption, which occurred near the end of the infusion period.

** Turnover rate (μEq/min.) was obtained in only 7 of the 11 cases. It was measured by maintaining a constant intravenous infusion of a tracer amount of 1-^{14}C-palmitate and measuring the specific radioactivity of plasma FFA at intervals. Turnover rate = radioactivity infused per minute (c.p.m./min.) divided by specific activity of plasma FFA (c.p.m./μEq). (Data of Steinberg, Nestel, Buskirk and Thompson, 1963.)

norepinephrine was similarly blocked by pronethalol, we gave our subjects an intravenous infusion of the drug just before the norepinephrine infusion. This effectively prevented the rise in FFA levels and turnover and at the same time blocked the increase in oxygen consumption (Table 10). The changes in pulse rate, blood pressure and respiration induced by norepinephrine were not appreciably influenced by prior pronethalol treatment. In other words the calorigenic action appears to be a true metabolic effect not attributable to increased cardiovascular or respiratory work.

Table 10. *Blocking effect of pronethalol on the increased FFA turnover and increased oxygen consumption induced by norepinephrine**

	Percentage change induced by Equal Dosage Rates of Norepinephrine			
	Control Period		After Pronethalol	
Patient	FFA Turnover	O_2 Consumption	FFA Turnover	O_2 Consumption
W.S.	+136	+19	−7	−2
D.C.	+72	+39	+1	+8
B.L.	+11	+25	−30	−15
R.B.	+27	+14	−35	−4
B.L.	—	+28	—	−4

* Subjects were given infusions of norepinephrine as described in the footnote to Table 9. While still in the metabolic chamber, 75–100 mg of pronethalol was infused by vein over a 10-minute interval and then the norepinephrine infusion was repeated at once at a dosage rate equal to that used in the control period. (Data of Steinberg, Nestel, Buskirk, and Thompson, 1963.)

It is, of course, still a possibility that the calorigenic action of norepinephrine is exerted by way of some unidentified mechanism and that pronethalol interferes with that at the same time that it blocks FFA mobilization. However, our data are compatible with the possibility that the increase in FFA mobilization is the immediate effect leading to increased oxygen consumption. Respiratory $^{14}CO_2$ production from labeled plasma FFA was also shown to be increased by norepinephrine in these clinical studies and this, too, was prevented by pronethalol, further supporting the above interpretation.

Finally, we would like to call attention to the association of high FFA levels with hypermetabolism in the hyperthyroid state. If elevated levels of FFA can, as suggested here, stimulate overall O_2 consumption, then it is possible that the hypermetabolic effect of thyroid hormone may, at least in part, be indirect. As discussed above, the rate of release of FFA from adipose tissue taken from hyperthyroid animals is elevated and the response of the adipose tissue to catecholamine stimulation is exaggerated (section III.C). Thus thyroid hormone, while it may have direct effects on oxygen consumption at the cellular level, may also influence oxygen consumption *indirectly* by causing overly rapid mobilization of FFA. If the exclusive effect were on rate of substrate utilization in the peripheral tissues, then the plasma levels of FFA might be expected to be *low*. The fact that they are *high* indicates some independent mechanism. The quantitative importance of the mechanism proposed (Eaton & Steinberg, 1961; Steinberg *et al.*, 1963) in determining the observed hypermetabolic state is under investigation.

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DISCUSSION

S. J. Folley: I am interested in the fatty acid cycle which you have described. Presumably in the lactating animal it would syphon out triglyceride from the cycle. Replenishment of fatty acids would occur by lipogenesis in the liver and the adipose tissue.

D. Steinberg: Yes, I think that would be analogous to the recently demonstrated ability of adipose tissue, a tissue of a less specialized type, to take up lipoprotein triglyceride. The mechanisms may of course be different but the analogy is a good one.

H. Bühring: We have studied the influence of different levels of fatty acids on the metabolism of glucose by muscle and find that on raising

the concentration of fatty acids in the medium used *in vitro* we obtain a decreased oxidation (decreased total utilization) of glucose. This is judged from the uptake of glucose and decreased turnover of glycogen.

D. Steinberg: If you added the fatty acid and glucose together, is the total substrate utilization changed in any direction?

H. Bühring: We kept the amount of the glucose in the medium constant, and the amount of fatty acids added was equivalent to normal values in rat plasma. When it was raised 4-fold we found a decrease in glucose utilization of about 50% and an increase in fatty acid oxidation of 180%. If you take into account the total amount of oxygen used for oxidation of the substrates it was slightly increased. Thus, if 29% were required for oxidation of fatty acids and glucose at a raised fatty acid concentration, about 20% was required when fatty acid and glucose remained in the normal state as they are in plasma.

D. Steinberg: Your results are very similar to those of Dr. Randall's group. He found the mutually reciprocal effect of added glucose and fatty acid on each other's oxidation. But I should like to know what happens to the total substrate utilization in order to correlate with possible changes in B.M.R. I am interested to hear that you did find some increase.

D. S. Robinson: I should like to stress the significance of the indirect transport to which you referred, namely the recirculation as triglycerides of mobilized free fatty acid. This may be of importance in providing for the specific needs of the different body tissues. The intake of free fatty acids, as you pointed out, is primarily determined by their concentration; this may not be sufficiently sensitive to control. The utilization of triglyceride, on the other hand, is determined by the clearing factor lipase action which may be more readily controlled. I wonder whether, in the fasting animal, you have any information on the relative importance of free fatty acids and triglyceride in contributing to body tissue metabolism.

D. Steinberg: I did not have time to discuss facts which bear on the question raised by Dr. Robinson. When we stimulate with catecholamine and get a 20% increase in oxygen consumption, the increase in the turnover of the fatty acid is 3 or 4 times as great. If we calculate how much oxygen would be needed to oxidize this extra fatty acid that is being turned over, it is far in excess of the extra oxygen the patient actually uses. In other words, a great deal of this increased turnover is fuss and feathers and getting nowhere. The fatty acids are being moved but they are not being oxidized. I think that a large proportion probably goes, as you suggest, to ester forms, some in the muscle, some in the liver. What goes into the liver probably comes out again as lipoprotein

triglyceride. Havel's studies certainly indicate that this is a very active and rapid system, at least with regard to the very low density lipoproteins. I do not know about the absolute contributions of free fatty acids in the basal state. I would not comment on it for the simple reason we do not know how valid our measure of fatty acid turnover is in an absolute sense. From the relative changes, however, it is clear that when you stimulate fatty acid turnover a large fraction of the extra turnover does not go to CO_2.

Jean Hagen: Is glycerol a particularly good substrate for triglyceride glycerol formation in muscle? How does it compare with, for example, glucose, and is there a glycerol kinase?

D. Steinberg: I do not know. Somebody here must know whether there is glycerol phosphokinase in muscle.

P. B. Garland: There is not. Neptune, using radioactive glycerol, has shown that the rat diaphragm will take up 5%. It may, however, have been exchanged. I have never been able to measure net glycerol uptake in the rat diaphragm. Wieland in Germany has published work on the glycerol kinase content of various tissues and he found none in heart, spleen or muscle.

W. M. Leat: Would it be true or false to say that if there is an increase in the level of free fatty acids in the serum the turnover rate is also increased?

D. Steinberg: Clearly it does not follow necessarily on any theoretical basis. You could have the concentration change in one direction and have the turnover rate go the opposite direction. In fact there is one good example and that is during exercise. Severe exercise causes a drop in serum free fatty acid concentration but the turnover is actually up. I do not know of an example where the concentration goes up and the turnover goes down, but I think I would approach it by making that my working hypothesis and check it.

R. Paoletti: In the perfused liver you show that triglyceride formation is dependent upon the concentration of free fatty acids in the perfusing medium. Do you observe the same when the liver is from an adrenalectomized animal?

D. Steinberg: I do not know.

R. Paoletti: I have observed that in adrenalectomized animals ACTH in large doses causes an increase in plasma-free fatty acid. It does not, however, increase liver triglycerides. A second question is about the so-called calorigenic action of thyroxine and its relation to free fatty acid levels in blood. Of course there are many other conditions, like diabetes,

where free fatty acid levels in blood are very high and apparently there is no calorigenic effect. Have you any comment on this?

D. Steinberg: Obviously I am not supposing that free fatty acid level is the universal thermostat that always determines the metabolic rate. Metabolic rate is a function of the amount of glucose, the amount of insulin, the blood flow, the body temperature, peripheral vasoconstriction, and so on. Clearly the free fatty acid level would not be expected to correlate directly on a 1 : 1 basis with metabolic rate, even though it might be a factor modulating metabolic rate under these circumstances. I might just add that it has been reported that the juvenile diabetic, who is a rather severe diabetic, does have an elevated basal metabolic rate, but obviously he is not using glucose.

N. B. Myant: We are very interested in Dr. Steinberg's last remarks about the possibility that a rise in the level of free fatty acids might be the thing that mediates the calorigenic action of thyroid hormone. I think he is less definite about this now than he was at one time. However, this is such an interesting matter than Miss Witney and I thought we would see what happened if we measured the earliest time at which we could detect a rise in the basal metabolic rate of rats after giving them thyroxine, and compared this with earliest time at which there was a detectable rise in plasma-free fatty acids. So far we have not been able to detect any rise in free fatty acid concentration until about 3 days after the beginning of a course of treatment. We can, however, just detect a rise in oxygen consumption after 1 day. Of course, it could be argued that we are in fact causing a higher rate of turnover of free fatty acids at an earlier time than we detect a rise in concentration. Another point is that if the free fatty acid level is so important in determining the basal metabolic rate, this should be extremely high in diabetes. These are really two separate points. Perhaps Dr. Steinberg could answer them.

D. Steinberg: The second point I thought I had answered to the effect that obviously many things determine metabolic rate. I think I listed seven or eight of them—body temperature, environmental temperature, peripheral vasoconstriction, heart rate and what have you, and more particularly the rate at which glucose is being utilized. In the diabetic who is not utilizing glucose at a normal rate the effect of free fatty acids would be difficult to predict. With regard to my degree of conviction about whether the free fatty acid level is an important determinant, I do not know whether I have said it more dogmatically than I should have in the text, I consider it a workable hypothesis in the light of what evidence I have and am aware of. In the investigations of Rich, Bierman and Schwartz (*J. clin. Invest.*, 1959, **38**, 275), large single doses of T3 were given and the rise in free fatty acid level certainly accompanied at least the rise in oxygen consumption. I think the approach that you are

taking is an interesting one and might lead to a definite answer, particularly if FFA turnovers were measured at the same time. I shall be interested to hear what results you get.

A. M. Barrett: Dr. Steinberg, you have shown that the administration of adrenaline in oil to dogs leads to a massive increase in plasma-free fatty acids and that if this treatment be continued for several days there is a 30% increase in plasma cholesterol and phospholipids. You suggest that the increases in cholesterol and phospholipid are a consequence of excessive free fatty acid mobilization. My own interests have been concerned with effects of catecholamines on the pituitary-adrenal axis and corticosteroid release. Now your doses of adrenaline induce large increases both in blood sugar and adrenocorticosteroid levels concomitantly with the rise in plasma-free fatty acids. Thorp and I have repeated your experiments and confirmed your results—in fact our cholesterol increases matched yours to within 0·1%! We extended the experiment by substituting oral glucose and subcutaneous ACTH for the adrenaline treatment. The fasting blood sugar was doubled, the plasma cortisol maximally elevated but plasma-free fatty acids were depressed to about 25% of the pre-dosing fasting level. After three days of this treatment the plasma cholesterol and phospholipids had increased by 28%.

You have also demonstrated hypercholesteraemia in the rat following adrenaline injections. We have found that 4 hours after subcutaneous injection of adrenaline in oil (2 mg/kg) plasma FFA increased from 372 to 1036 μEq/l, blood sugar from 116 to 291 mg% and plasma corticosterone from 6·6 to 34·7 μg/100 ml. Yet after three days of this treatment there was no significant change in plasma cholesterol concentration. All we showed was a fall in phospholipid and hence C/P ratio but this was subsequently seen as part of a weekly pattern, phospholipids falling from Monday to Friday. It would seem therefore that cholesterol increases can follow decreased FFA mobilization in the dog or fail to follow increased FFA release in the rat. I wonder if you have any comment?

D. Steinberg: The results with ACTH are most interesting. I should say personally that if any agreement is within 0·1% it is highly suspicious! Zilversmit, in a careful study, conducted some time ago, of the effect of ACTH on dogs, found that if you took an adrenalectomized animal whose serum lipids had fallen, treatment with cortisone would bring them back up. ACTH or cortisone given to a normal dog had very little effect on basal blood lipid levels. The difference between their experiment and yours is that you gave large doses of oral glucose—is that correct? It would seem to be in quite a different category—perhaps related in some way to what we now recognize as carbohydrate-induced lipaemia, and I would ask you to put on the record whether the triglyceride levels were elevated out of proportion to the other lipids.

A. M. Barrett: We did not estimate the triglyceride directly but calculated them by a difference method and obtained equivocal results. I would not like to be definite about triglyceride in these experiments, but there were no disproportionate increases nor was there any visible lipaemia in the plasma.

THE INFLUENCE OF THYROID HORMONE UPON THE SYNTHESIS OF FATTY ACIDS IN THE LIVER

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If rats are injected with thyroxine there is a depression of fatty acid synthesis in slices of their livers incubated *in vitro* (Fletcher & Myant, 1958). In this paper we shall consider how thyroxine brings about this effect.

First, however, it should be mentioned that treatment with thyroid hormone has been shown to increase the incorporation of hydrogen deuterium oxide (HDO) (Karp & Stetten, 1949) and of [^{14}C]-acetate (Dayton, Dayton, Drimmer & Kendall, 1960) into liver fatty acids *in vivo*. We shall not discuss this contradiction here, except to point out that variable factors, such as the amount of endogenous precursor present and the rate of turnover of the labelled product, will influence the net incorporations of a labelled precursor to a greater extent if the experiment is done *in vivo* than if it is done *in vitro* with isolated tissues incubated for short periods. Nor can it be assumed that all the labelled product found in a given tissue in the *in vivo* experiment was actually synthesized *in situ*. It is by no means certain, therefore, that the findings of Karp & Stetten and of Dayton *et al.* give a true indication of the effect of thyroxine upon fatty acid synthesis in the liver.

A decrease in fatty acid synthesis in liver slices can be detected about 24 hr. after a single injection of thyroxine, provided that the rats are of the same sex, of an inbred strain, and are killed at the same time of day. Figure 1 shows the results of an experiment in which synthesis of fatty acids from [1-^{14}C]-acetate was measured in slices of liver at intervals during a series of daily injections of thyroxine. For reasons to be discussed later, the concentrations of ATP and glycogen in the liver were also measured. The fall in fatty acid synthesis was just detectable 24 hr. after the first injection, and by the fourth day it had reached its full extent. The change in fatty acid synthesis was always associated with a fall in the concentrations of ATP and glycogen in the liver. Of the three effects shown in Fig. 1, the most marked, and possibly the earliest, is a fall in liver glycogen. Within 18 hr. of a single injection of thyroxine, the concentration may fall to 50% of the level in untreated rats. A curious finding, already noted by Parhon (1913),

is the almost complete absence of an effect of thyroxine upon muscle glycogen (Fig. 1).

The possibility that the fall in fatty acid synthesis might be mediated by changes in the supply of intracellular cofactors led Fletcher &

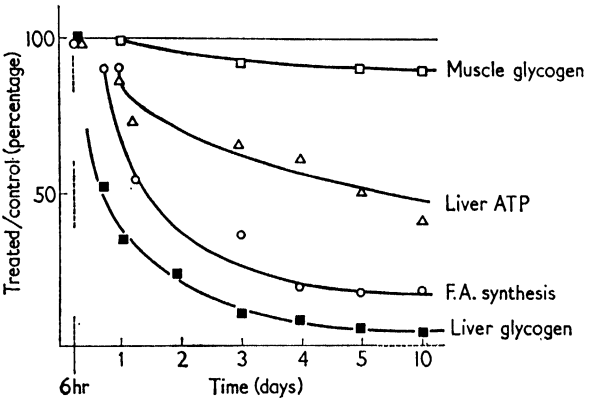


Fig. 1. Incorporation of [1-^{14}C]-acetate into fatty acids in liver slices (○), glycogen content of liver (■) and thigh muscle (□) and ATP content of liver (△) at intervals after the beginning of a series of 24-hourly intraperitoneal injections of L-thyroxine (50–100 μg./day) into rats. First injection at zero time. Results shown for treated rats, expressed as percentages of values in untreated rats killed at the same intervals. Methods described by Fletcher & Myant (1958, 1961*b*.) (Fletcher & Myant, unpublished work.)

Myant (1960) to test the effect of thyroxine treatment upon fatty acid synthesis in cell-free fractions of liver from which the heavier subcellular particles, including the mitochondria, had been removed by centrifugation at 10,000 g. (S_{10} fractions).

It was first necessary to find out how far the fatty acids in this preparation are synthesized by way of malonyl-CoA. In this pathway (Wakil, 1961), malonyl-CoA is formed by the carboxylation of acetyl-CoA – a reaction which requires carbon dioxide, ATP and acetyl-CoA carboxylase. In the subsequent steps, which require NADPH but not ATP, units of malonyl-CoA condense successively with a priming unit of acetyl-CoA to form long-chain fatty acids; the complex of enzymes catalyzing these steps is sometimes known as malonyl-CoA synthetase.

In the S_{10} fraction from normal rat liver at least 90% of the fatty acid synthesized from acetate was shown to be formed by way of malonyl-CoA (Fletcher & Myant, 1961*a*). More recently (Iliffe & Myant, unpublished), we have found that the capacity of this preparation for incorporating malonyl-CoA into fatty acids (in μmoles/g. liver protein) is at least ten times that for incorporating acetate; this suggests that the rate-limiting step for fatty acid synthesis in the S_{10} fraction lies between acetate and malonyl-CoA.

Treatment of the rats with thyroxine was found to lead to a depression of fatty acid synthesis from acetate in the S_{10} fraction, and this

fect was associated with an absence of particulate glycogen from the reparation. The depression could be reversed by adding glycogen or TP at high concentrations *in vitro*. Further, when the particulate ycogen was removed from S_{10} fractions prepared from untreated ats, fatty acid synthesis was depressed. Table 1 shows the results of a pical experiment illustrating these relationships between glycogen nd the synthesis of fatty acids. We have since found that synthesis of tty acids from malonyl-CoA in S_{10} fractions is not depressed signi-cantly by treating the rats with thyroxine, even when there is marked epression of synthesis from acetate (Table 2).

able 1. *Incorporation of [1-^{14}C]-acetate into fatty acids in liver S_{10}; fractions epared from normal and thyroxine-treated rats; influence of glycogen upon acetate incorporation into S_{10}; fractions from normal and treated animals*

S_{10} fraction	Acetate incorporated
Control	100
Treated	21
Treated + glycogen (30 mg/ml)	107
Control without particulate glycogen	47

ne treated fractions were prepared from the pooled livers of 6 rats, each given 50 μg. thyroxine daily for 9 days. The control fractions were prepared at the same time from untreated rats. For conditions of incubation see Fletcher & Myant (1960). Acetate corporated into fatty acids expressed as percentage of incorporation in control S_{10} action. (Fletcher & Myant, unpublished work.)

able 2. *Incorporation of [1-^{14}C]-acetate (2·5 mM) and of [2-^{14}C]-malonyl-CoA <0·1 mM) into fatty acids in S_{10}; fractions of liver from normal rats and from rats given 100 μg. L-thyroxine daily for 3 days*

	Homogenate	Acetate incorporated	Malonyl-CoA incorporated
Control	1	0·30	27·4
	2	0·39	18·3
	3	0·35	24·7
Thyroxine treated	4	0·07	21·9
	5	0·08	21·2
	6	0·05	25·7

nditions of incubation described by Fletcher & Myant (1960). For this experiment, separate homogenates were prepared, 3 from normal and 3 from treated rats. Values pressed as percentage of added radioactivity incorporated into fatty acids. (Iliffe, yant & Witney, unpublished work.)

Investigation of the rôle of glycogen as a source of energy for the generation of ATP during incubation of the S_{10} helped to provide an explanation for these findings (Fletcher & Myant, 1961*b*). It was found (Fig. 2) that the ATP level fell rapidly in the S_{10} fraction from treated ats unless glycogen was added at the beginning of the incubation, and hat if glycogen was added half way through the incubation there was a apid rise in ATP concentration. In the S_{10} fraction from untreated rats,

on the other hand, the ATP level remained close to the initial level until near the end of the incubation. These results show that the maintenance of a steady concentration of ATP in the S_{10} fraction is the net result of continual breakdown and regeneration, and that the energy required

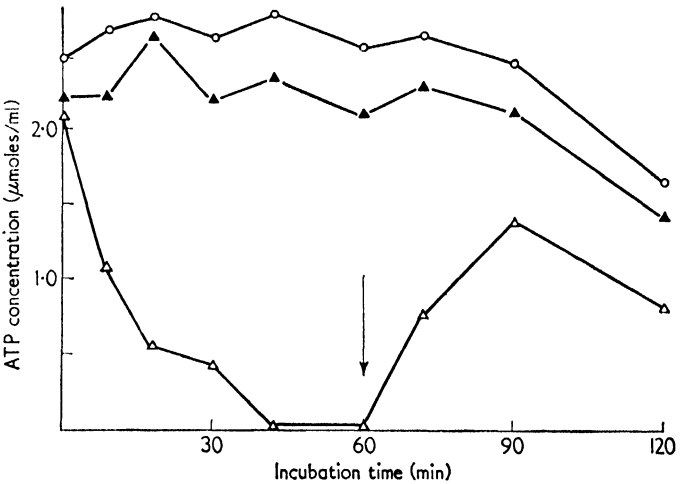


Fig. 2. Effect of glycogen upon ATP level in S_{10} preparations from normal and thyroxine-treated rats during 2-hr. incubation at 37°. ATP concentration in control S_{10} fraction (○), in treated S_{10} to which glycogen (30 mg/ml) was added at zero time (▲), and in treated S_{10} to which glycogen (10 mg/ml) was added at end of 1 hr. (△). Exogenous ATP (2 μmole/ml) was added to all three incubation flasks at zero time. (Adapted from Fletcher & Myant, 1961*b*.)

for regeneration is derived from glycogen. In the absence of glycogen, therefore, the ATP present at the beginning of the incubation disappears rapidly. The significance of the fall in ATP concentration in the S_{10} fraction from treated rats became clear when the relationship between ATP concentration and fatty acid synthesis was studied in control preparations from which the endogenous ATP had been removed by dialysis. In the absence of added ATP no synthesis occurred. As the ATP concentration was raised, synthesis rose steeply to a maximum at 2–3 mM ATP and then declined slightly at higher concentrations (Fig. 3).

On the basis of these findings it was suggested that treatment with thyroxine depresses fatty acid synthesis from acetate by diminishing the glycogen content of the preparation and thus leading to a failure to regenerate ATP. On this view, it is not the initial concentration of endogenous ATP in the S_{10} fraction that determines the amount of fatty acid synthesized *in vitro*, so much as the ability of the preparation to maintain the concentration at an adequate level for a period of two or more hours. It is implicit in this explanation that the rate-limiting step in fatty acid synthesis from acetate in the S_{10} fraction is one that requires ATP. As we have already noted, the available evidence

suggests that this is so. The finding that thyroxine does not influence the incorporation of malonyl-CoA into fatty acids (Table 2) is also consistent with this hypothesis, since ATP is not required once malonyl-CoA has been formed.

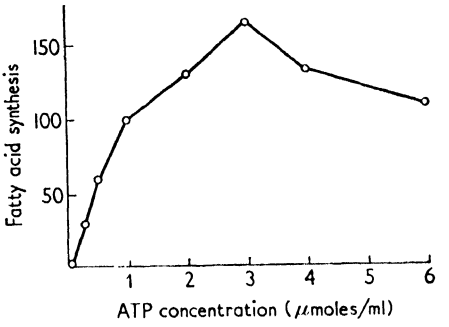


Fig. 3. Incorporation of [1-^{14}C]-acetate into fatty acids in dialyzed S_{10} preparation in the presence of increasing concentrations of ATP. The incubation mixture contained exogenous $NADP^+$, NADH, CoA and glycogen. Values expressed as percentage of incorporation observed in the presence of 1 mM ATP. (Adapted from Fletcher & Myant, 1961*b*.)

When the above explanation was put forward, it was suggested that there might also be a slight shortage of NADPH in the treated preparations, on the grounds that fatty acid synthesis in the treated S_{10} fraction was stimulated by the addition of isocitrate. However, it has now been shown that stimulation of fatty acid synthesis by citrate and isocitrate cannot be explained by an increase in the supply of NADPH (Abraham, Matthes & Chaikoff, 1960; Martin & Vagelos, 1962; Waite & Wakil, 1962).

The question now arises: how far are these observations on subcellular fractions relevant to what happens within the intact liver cell?

If fatty acid synthesis in the whole cell is related to ATP concentration in the same way as it is in the S_{10} fraction, then the fall in synthesis observed in liver slices from thyroxine-treated rats could be explained largely, if not entirely, by the fall in ATP content. The concentration of ATP in normal rat liver is about 2·4 μM/g., and after treatment with thyroxine it falls to less than 1 μM/g. Figure 3 shows that in the S_{10} fraction a change in ATP concentration of this magnitude would cause a fall in fatty acid synthesis to less than 50% of the maximal level. How thyroxine brings about a fall in the ATP content of the liver *in vivo* is still an open question. It used to be thought that the effect was due to inhibition of oxidative phosphorylation in the mitochondria, but there is some doubt as to whether this can explain the effects of small doses of thyroxine close to the physiological range (Fletcher, Myant & Tyler, 1962). It should be noted that, in suggesting an explanation in terms of ATP concentration, we are assuming that the rate-limiting step in the

whole cell, as in the S_{10} fraction, is one for which ATP is required. More will be said about this below.

In the S_{10} fraction, glycogen was found to have an important rôle in maintaining the concentration of ATP, upon which fatty acid synthesis from acetate depends. In the intact respiring liver cell, on the other hand, ATP is usually assumed to be regenerated by oxidative phosphorylation in the mitochondria. We might, therefore, be tempted to regard the rôle of glycogen in the S_{10} fraction as significant only in so far as it shows how fatty acid synthesis can be regulated *in vitro* by the supply of ATP, and not as representing a physiological mechanism. On the other hand, there are several other instances, besides that of thyroxine treatment, where a change in fatty acid synthesis is closely correlated with a change in glycogen content (see Masoro, 1962). For this reason it has been suggested that glycogen itself exerts some more or less specific influence upon fatty acid synthesis in the whole cell. A correlation between glycogen level and fatty acid synthesis is no proof that the one influences the other. Nevertheless, glycogen could, perhaps, affect the supply of ATP within the cell by providing substrate for mitochondrial oxidation. It might also affect the supply of NADPH, though this should only influence the rate of synthesis of fatty acids in conditions in which the steps beyond malonyl-CoA become rate-limiting.

Some of the difficulties that arise when one tries to deduce what happens in the whole cell from observations made on subcellular fractions raise questions of general interest. One of these questions concerns the rate-limiting step in a biosynthetic sequence. If one wants to know how a hormone influences a biosynthesis in the intact cell, it becomes important to find out which is the rate-limiting step and which are the factors that influence it, since this is the step whose rate must control the rate of the whole sequence. It is not always recognized, in spite of what Chance (1961) has said, that the step which becomes rate-limiting in a given set of conditions *in vitro*, when enzymes and cofactors may be added in unphysiological concentrations, is not necessarily the rate-limiting step *in vivo*. Nor is it likely that the same step will be rate-limiting *in vivo* in all conditions. It is obvious, for example, that although the carboxylation of acetyl-CoA may well be rate-limiting for fatty acid synthesis *in vivo* under some conditions, the steps beyond malonyl-CoA would become rate-limiting if the supply of NADPH were reduced to a low enough level or, as Langdon (1960) has suggested, if other reactions competing for NADPH became sufficiently active. It does not follow, therefore, that because an ATP-requiring reaction is rate-limiting in the artificial environment of the S_{10} fraction, it is also rate-limiting under all conditions in the whole cell. Nor does it follow that it is always the supply of ATP which determines the rate of the carboxylation reaction; the supply of CO_2 could become rate-limiting if it fell to a low enough level, or, again, if reactions competing for

carbon dioxide became active enough. The extent to which enzyme activity becomes rate-limiting is likewise difficult to deduce from *in vitro* experiments. The fact that in several mammalian tissues the activity of acetyl-CoA carboxylase is less than that of malonyl-CoA synthetase, when both enzymes are assayed in the presence of cofactors at optimal concentrations (Ganguly, 1960), is certainly suggestive, but it does not prove that the carboxylation of acetyl-CoA is rate-limiting for fatty acid synthesis *in vivo*, since it cannot be assumed that cofactor concentrations are optimal in the intact liver cell. The same criticism may be made of the suggestion of Numa, Matsuhashi & Lynen (1961) that the depression of fatty acid synthesis in the livers of starved animals is due to the fall that occurs in the activity of acetyl-CoA carboxylase. The fall in enzyme activity may be an adaptation to, rather than the cause of, the diminished rate of fatty acid synthesis, which may itself be mediated by some other factor, such as a fall in ATP concentration.

With these considerations in mind, we have begun to study a rat-liver homogenate which resembles the normal cell more closely than the S_{10} fraction in two respects: it has no exogenous cofactors, and its supply of ATP is derived entirely from phosphorylating mitochondria. This preparation synthesizes long-chain fatty acids from acetate via the malonyl-CoA pathway. The supply of ATP is rate-limiting for the overall incorporation of acetate into fatty acid, and in the presence of non-limiting concentrations of substrate the preparation is capable of synthesizing several times as much fatty acid from malonyl-CoA as from acetate. Thus, in conditions which are more physiological than those of the S_{10}, the rate-limiting step appears, as in the S_{10}, to be earlier than the formation of malonyl-CoA, and may, therefore, be the carboxylation of acetyl-CoA.

The properties of this preparation favour the suggestion of Fletcher & Myant (1961*b*) that the supply of ATP is rate-limiting for fatty acid synthesis in liver slices. On the other hand, our findings argue against the suggestion of Langdon (1957) and of Siperstein & Fagan (1957) that the step upon which insulin and, perhaps, other hormones act indirectly to modify fatty acid synthesis is one requiring NADPH. However, as we have already pointed out, even if it could be proved that the carboxylation reaction is normally rate-limiting in the intact cell, this would not exclude the possibility that in some conditions the reactions beyond malonyl-CoA become rate-limiting.

ADDENDUM

Finally, I should like to refer to the effect of tricarboxylic acids upon fatty acid synthesis in our mitochondrion-containing preparation, since this provides another example of the difficulty of extrapolating from particle-free systems to intact cells. Citrate and isocitrate have been

shown to stimulate fatty acid synthesis in particle-free preparations of liver, mammary gland and adipose tissue; nearly 60-fold stimulation has been observed in some cases. Working with a purified enzyme, Vagelos, Alberts & Martin (1963) have recently shown that this effect is due to enhancement of the activity of acetyl CoA carboxylase, perhaps by alteration of its state of molecular aggregation. Accordingly, they have suggested that the intracellular concentration of citrate is an important factor in the regulation of fatty acid synthesis in the normal cell. This hypothesis would only be acceptable if one could be sure that the enzyme is sensitive to activation by tricarboxylic acids when it is in its natural state within the unbroken cell.

In our system we have been unable to demonstrate a stimulatory effect of isocitrate or citrate comparable with the effect observed in more highly purified systems. Table 3 shows the effect of adding isocitrate or citrate to the preparation. Isocitrate stimulated acetate incorporation slightly at 5 and 10 mM, but at higher concentrations it was

Table 3. *Effect of isocitrate and citrate upon incorporation of [^{14}C]-acetate into fatty acids in whole homogenate containing phosphorylating mitochondria*

Additions (mM)	[1-C]-acetate incorporated
None	100
Isocitrate (5)	126
,, (10)	111
,, (20)	44
,, (5 + 5)*	97
ATP (2), no isocitrate	472
ATP (2), isocitrate (20)	314
Citrate (15)	50

* Second addition of isocitrate after 1 hour. Incubation mixture = Whole homygenate in tris/KCl, pH 7·4; acetate, 2·5 mM; PO_4, 1·0 mM; Mg^{2+}, 4 mM; succinate, 15 mM; no added cofactors.

inhibitory. Citrate was inhibitory at all concentrations tested. In order to exclude the possibility that isocitrate failed to stimulate incorporation because it was oxidized before any activation of acetyl CoA carboxylase could take place, we tried the effect of adding isocitrate in two doses, one at the beginning of the incubation and one an hour later. This had no stimulatory effect. Nor was isocitrate stimulatory in the presence of 2mM. ATP, the concentration of ATP which we have found to be optimal for acetate incorporation into fatty acids in this preparation.

Interpretation of these results is complicated by the fact that the preparation must contain the citrate cleavage enzyme, which could form non-radioactive acetyl CoA from the added citrate. This might mask a small stimulatory effect by depressing the incorporation of radioactive acetyl CoA formed from the ^{14}C-acetate used as precursor. If any of the added isocitrate were converted to citrate, a lowering of the specific activity of the pool of acetyl-CoA could arise in the same way.

Nevertheless, our results raise the possibility that acetyl CoA carboxylase in the unbroken cell, and in the whole homogenate, is not activated by isocitrate or citrate, and that it becomes sensitive to their action only during the preparation of particle-free systems. It is perhaps worth noting that the activating effect of tricarboxylic acids in purified enzyme preparations is very slight at concentrations below 1 mM. (Martin & Vagelos, 1962), whereas the concentration of citrate in normal rat liver is about 0·1 mM. (Frohman, Orten & Smith, 1951).

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DISCUSSION

J. M. Lowenstein: I agree with Dr. Myant's ideas about the activation of the acetyl coenzyme A carboxylase. The physiological citrate concentrations that we have been able to measure in liver and mammary gland all fall in the range of 0·3 to 0·5 mM. These are far from enough to activate the carboxylase. Under physiological conditions the concentration of isocitrate would be expected to be about 1/10 that of citrate, by virtue of the aconitase equilibrium, which favours citrate over isocitrate in the ratio of about 9 to 1. So if these tricarboxylic acids are the activators, there is not sufficient of them in liver and mammary gland to activate the enzyme fully. I share your view that the enzyme is fully activated in intact cells but by something else. I do not regard the activation as an artefact, because we have looked at this in very freshly prepared systems—that is, 1 or 2 minutes after making the homogenate

and have found that the activity of the carboxylase has already been lost. I would suggest the possibility that in your system the limiting factor is the acetate-activating enzyme.* Perhaps the reason why you do not get any activation on adding citrate is that the acetate-activating enzyme is limiting. The activity of this enzyme is very low in normal livers.

N. B. Myant: Do you think it likely that citrate would be incorporated into fatty acids in our system?

J. M. Lowenstein: Yes. As a matter of fact we get faster incorporations of citrate than we do of acetyl coenzyme A. This seems a paradox but it appears that there are other reactions of acetyl coenzyme A including hydrolysis, so that if you add acetyl coenzyme A in substrate amounts, you tend to lose it, but if you add citrate it generates acetyl coenzyme A much faster than it is lost by interfering reactions.

G. Popják: Was the effect of malonate, instead of isocitrate or citrate, tested in the complete homogenate?

J. Iliffe: Yes. Malonate has no stimulatory effect.

N. B. Myant: In fact, we always found inhibition of acetate incorporation into fatty acids with malonate concentrations above 5 mM. However, the interpretation of experiments with malonate in our system is rather difficult, because the supply of ATP is dependent entirely upon phosphorylating mitochondria. Part of the inhibitory effect of malonate is undoubtedly due to a lowering of the ATP level. Another complication is that malonate is itself incorporated into fatty acids in our system, perhaps by activation to malonyl-CoA, so that if non-radioactive malonate is added, this may lower the specific activity of the radioactive malonyl-CoA arising from radioactive acetate.

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* Note added in proof by Myant & Iliffe. This explanation seems unlikely, since incorporation of acetate in the homogenate could be stimulated markedly by the addition of ATP (Table 3).

THE LIPOLYTIC EFFECT OF GLUCOCORTICOIDS

By R. F. MAHLER and WINIFRED L. STAFFORD

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HE antilipolytic effect of insulin is generally attributed to its ability) enhance glucose utilization and therefore to re-esterify liberated free ıtty acids. Similarly, Fain, Scow & Chernick (1963), who have ›cently shown *in vitro* that the glucocorticoids, dexamethasone and ›rticosterone, promote lipolysis, suggest that this effect is secondary › the accompanying decrease in utilization of glucose.

We wish to report that *in the absence of glucose*, lipolysis is increased y addition *in vitro* of cortisol (equivalent to 30 μg/ml) to rat epididymal ıt in four situations: (a) unmodified, (b) treated with norepinephrine, ›) treated with insulin, and (d) treated with both norepinephrine and ınsulin (Jungas & Ball, 1962).

Pieces of tissue were incubated for 90 minutes in Krebs bicarbonate uffer containing 3% crystalline bovine albumin but no glucose (glu›se content 0·03 μg/ml). The extent of lipolysis was inferred by nalysis for glycerol released into the medium, using the periodate xidation method of Van Handel & Zilversmit (1957).

able 1. *Effect of cortisol (30 μg/ml) on glycerol release from rat epididymal tissue*

Tissue from each of 8 rats appeared in each of the 8 groups. Norepinephrine at 0·09 μg/ml, insulin at 90 μU/ml. Incubation time 90 minutes.

Additions	Without cortisol μmoles/g/90 min.*	With cortisol† μmoles/g/90 min.*	Δ Glycerol μmoles/g/90 min.*	Values of P
one	1·67 ± 0·15	2·00 ± 0·08	+0·33 ± 0·32	<0·05
orepinephrine	2·58 ± 0·35	3·68 ± 0·27	+1·10 ± 0·36	<0·05
orepinephrine and Insulin	1·78 ± 0·30	2·87 ± 0·15	+1·09 ± 0·41	<0·05
ısulin	1·58 ± 0·18	1·92 ± 0·24	+0·34 ± 0·10	<0·01

* Mean value ± standard error of the mean.

† Sodium salt of the hemisuccinate = 30 μg cortisol/ml.

The results are shown in Table 1. In the absence of cortisol, norpinephrine significantly stimulated the release of glycerol. This was lmost completely inhibited when insulin was also present, while inılin by itself had no significant action. By contrast, in the presence of

cortisol lipolysis was increased in all four situations, and the rise did not differ significantly between them. When the two series were compared, the size of the glycerol release induced by norepinephrine and the inhibition of this effect by the simultaneous presence of insulin also did not differ significantly.

These findings indicate that cortisol can stimulate lipolysis in the absence of glucose, whether or not the tissue lipase system is activated by norepinephrine, or is under the anti-lipolytic influence of insulin. In addition, the observation that the relative effects on lipolysis of norepinephrine and insulin are unaltered by the presence of cortisol, suggests that cortisol influences the lipolytic system in a manner independent of the other two hormones.

Those who would postulate that all hormonal influences on lipolysis are mediated through an effect on glucose metabolism have been disturbed by the apparently anomalous position of catecholamines which stimulate both glucose uptake and lipolysis. The above findings suggest that both glucose-dependent and glucose-independent influences may exist. With glucocorticoids we may suppose that the two pathways act in the same direction, to promote lipolysis, but that with the catecholamines they oppose one another. Apparently, then, the glucose-independent stimulation of the lipase predominates with catecholamines even when glucose is available for re-esterification.

We may conclude that this stimulation is stronger than the stimulation by cortisol, resulting in the well-known greater lipolytic influence of catecholamines than the glucocorticoids.

ADDENDUM

Recently, one of us (W.L.S.) has been able to show a lipolytic effect of cortisol at a concentration of 0·3 μg/ml. provided that the rats used have been fed rat cake supplemented with 5% corn-oil. Further, the lipolysis was maintained in presence of fluoride, which inhibits the glycerol releasing action of catecholamines.

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The work was done during the tenure of a Medical Research Council Grant to W. L. Stafford.

DISCUSSION

D. Steinberg: Several groups have now shown that it is possible to stimulate the release of fatty acids from isolated adipose tissue with one or another of the cortical steroids. I think that in most cases the

concentrations used were higher than those Dr. Stafford found to work and raise the question of the physiological importance of this stimulant to fatty acid release. I would just like to mention results that we got with dogs given cortisone at about 5 mg per kg and somewhat lower. Administration of this caused no exceptional rise in free fatty acid levels in the blood. We did not have turnovers and cannot be sure that it was completely inactive. I want to make it clear, however, that in that same group of experiments we showed that the action of epinephrine on fatty acid release from the adipose tissue would only occur at a maximum rate provided the animals had their own sources of corticosteroids in the form of an intact adrenal cortex or that they were given cortisone. This was shown further to be related to a direct effect on the sensitivity of the adipose tissue in rats previously adrenalectomized. Their epididymal fat pads were not as responsive to epinephrine as those of control animals. This is confirmed by work in Jerusalem by Rocheck and Shapiro and I think others have found the same. Thus it would appear that there really are two categories of corticosteroid effects with regard to fat metabolism, perhaps one being the direct effect demonstrated here, the other being a potentiating or permissive effect, such as I have described. Would you agree that these may be two different sources of stimulation?

W. L. Stafford: Yes. My rats already had sufficient endogenous corticosteroid to provide basal, permissive conditions.

D. Steinberg: This is superimposed on the normal output of steroids. We also tried to get a potentiation of the epinephrine effect with corticoids *in vitro* but were unable to show this with the steroid added directly. In most experiments we did not get any increase in fatty acid release with the cortisone alone. But we may not have used the right conditions.

G. H. Sloane-Stanley: Are we to take it that glucocorticosteroids decrease the utilization of glucose by promoting lipolysis?

W. L. Stafford: I do not think we can say we have established the *primary* effect of cortisol to be lipolytic in fatty tissue. The fact that it is promoted *in vitro* in absence of a glucose substrate does not necessarily mean that it is the predominating one *in vivo*. This is one of the difficulties about hypothesizing from *in vitro* experiments.

G. Feuer: The main difference of corticosteroids in the different bloods are seen principally in the rat where the main component is corticosterone and in the human blood where the main component is cortisol. Have you got any experiment when you used cortisone for the same purpose? I should also like to know if it is the free or the protein bound cortisol in plasma which is the more effective.

W. L. Stafford: Steroid was added in the free form to a medium containing crystalline bovine serum albumin (when used at 30 μg/ml). At the lower concentration the medium was initially protein free.

There is the question of the increased sensitivity to cortisol of rats fed a diet supplemented with corn-oil. What their natural gluco-corticoid level is I have not determined but their tissues may be expected to contain more unsaturated acids, particularly linoleic and arichidonic acids, than normal.

THE RATE OF TURNOVER OF GLYCEROL IN BLOOD

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THE concentration of glycerol in the blood of rabbits is normally between 5 to 25 μmoles per 100 ml (Hagen & Hagen, 1962). In the fasting animal this glycerol probably arises principally from two sources, (1) hydrolysis of triglycerides in the adipose tissue, and (2) hydrolysis of the triglycerides of blood lipoproteins at the site of their removal from the blood. Since injected glycerol disappears very quickly from the blood (Hagen, 1963a) it seems likely that the small amount of glycerol in blood turns over quite rapidly. Experiments have now been done to determine the rate of turnover of blood glycerol and its metabolic fate (Hagen, 1963b).

[1,3-^{14}C]-Glycerol was infused intravenously into phenobarbital anaesthetized rabbits at a constant rate for 30 or 40 minutes. Samples of arterial blood were obtained at intervals during and after the infusion. Glycerol was isolated from the plasma as the tribenzoate, glucose was isolated as glucosazone and plasma lipids were extracted with chloroform: methanol mixture and washed (Folch, Lees & Sloane-Stanley, 1957). Expired carbon dioxide was collected in sodium hydroxide during fifteen-minute periods throughout the experiment and subsequently converted to barium carbonate. At the end of the experiment samples of liver and adipose tissue were removed and liver glycogen, liver lipid and adipose tissue lipid were isolated. The radioactivity of all compounds was measured in a low-background Nuclear Chicago gas-flow counter. The rate of turnover of glycerol was calculated from the rate of dilution of the infused [^{14}C]-glycerol by endogenous unlabeled glycerol (see Armstrong, Steele, Altzuler, Dunn, Bishop & De Bodo, 1961).

In four experiments the half-life of blood glycerol was 3·10, 2·08, 1·93 and 2·73 minutes. The rate of glycerol production in the rabbit was roughly proportional to the concentration of glycerol in the blood (8·49, 3·84, 3·01 and 12·80 μmoles per minute; 21·3, 8·3, 5·8 and 24·0 μmoles per 100 ml). Glycerol label was incorporated in appreciable amounts into plasma glucose, plasma lipid, liver lipid and respiratory carbon dioxide. Of the label in the liver lipid about half was in neutral

lipid and half in phospholipid. Very little label was incorporated into liver glycogen or adipose tissue lipid.

Although glycerol is present in very low concentrations in blood it appears to turn over rather rapidly. The concentration of glycerol in blood is influenced by a number of factors: it is increased by injection of noradrenaline (Hagen & Hagen, 1962; Schwarz, Eymer, Kopetz & Weinges, 1961) and in diabetes (Hagen, Moorhouse & Steinberg, 1963); it is decreased by insulin (Hagen, 1963a). The rate of entry of glycerol into the blood probably depends on both the rate of turnover of adipose tissue triglyceride and the rate of turnover of plasma lipoproteins; thus, any factor influencing either of these two processes would be expected to alter the concentration of glycerol in blood.

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DISCUSSION

A. M. Barrett: I believe that you have said, in a previous paper, if not in your present communication, that glucose caused a fall in glycerol in the plasma of ketotic diabetic patients when administered orally. Is this correct?

J. H. Hagen: Yes, it does.

A. M. Barrett: In collaboration with Dr. W. H. W. Inman, I have followed plasma free fatty acid and blood sugar levels in a series of five insulin dependent diabetics after a conventional oral glucose tolerance test. In none of these subjects have we observed any significant change in the elevated fasting free fatty acid (FFA) during the period of hyperglycaemia. In non-diabetic subjects the oral glucose tolerance test produces a marked fall in FFA to less than 50% of the fasting level. We had concluded that the fall in FFA induced by hyperglycaemia was insulin-dependent. Presumably the fall in plasma glycerol, which you have described, may be interpreted as an inhibition of lipolysis. If this is the case one might predict that your patients would also show a fall in plasma FFA. Have you found insulin-dependent diabetics to show a normal FFA response to hyperglycaemia? If not, does this imply that

FFA changes after glucose are dependent upon insulin release but that the effect on glycerol is not mediated by insulin?

J. H. Hagen: No, they do not give a normal response, not with the free fatty acids, but the fall in blood glycerol seems to be pretty nearly normal. I have no explanation for it; it may be that there is some stimulation of uptake of glycerol rather than an inhibition of release of glycerol.

D. Steinberg: Have you made any direct comparisons on the same animal or in different animals of turnover of glycerol and turnover of fatty acids? What is the relationship between the turnover rate of fatty acids?

J. H. Hagen: I have not studied the rate of fatty acid turnover.

D. Steinberg: In speculating about the role of glycerol one can quickly calculate that the amount of glycerol that is released as a result of triglyceride turnover in adipose tissue is quite considerable. At a very minimum, it has to be at least one-third the molar amount of fatty acid that is released from the adipose tissue, and so if you have somebody mobilizing fats as the sole source of calories, completely fasting, he mobilizes perhaps 200 g of triglyceride. Now 200 g of triglyceride corresponds to about 10% as much, namely 20 g of glycerol which on a calorie basis is certainly enough to support cerebral metabolism, suggesting a role for this stored glycerol which we tend to ignore. We always talk about the fat stores, forgetting that there is quite a lot of glycerol stored in adipose tissue, indeed a tremendous amount; 10 kg of fat, means about 1 kg of glycerol. This might be available as a source for the glucose without which the brain cannot survive. In a starving individual, the only other source is glucogenesis from protein.

J. H. Hagen: Yes, I have considered that. In fact I did a similar calculation for the rabbit based on the glycerol concentration and the glycerol turnover rate. In the normal rabbit I got something like 10% of the calories from glycerol. Now, of course, in the starved animal which was mobilizing fat it might be considerably greater.

EFFECTS OF SEASONAL VARIATION ON LIPID METABOLISM IN ANIMALS AND MAN

By J. M. THORP

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THE interaction between man and his environment is an uncontrollable variable which may provoke the lyric inspiration of a spring day or the respiratory distress of an autumn fog. By associating the former with hypersecretion of gonadotrophins, and the latter with hyposecretion of adrenocortical steroids, a biochemist could feel that he has placed his finger on the pulse of the seasons. If his interest lay in the factors regulating lipid metabolism, he would justifiably enquire how these varied with seasonal changes in environment (meteorological, dietary or social) and with associated changes in endocrine function. The nature of species and seasonal variations in the response of serum lipids to treatment with the compound ethyl α-(4-chlorophenoxy)-isobutyrate, (I.C.I. 28,257: Thorp & Waring, 1962), provided the basis for such an analysis. Evidence for the close similarity of patterns of seasonal variation of the circulating lipids (and the underlying metabolic processes they reflect) with variations in endocrine function is presented for discussion.

SEASONAL VARIATIONS OF SERUM LIPIDS

The broad patterns of seasonal variation of serum cholesterol (Fig. 1A) and plasma free fatty acid (Fig. 1B) in the rat followed closely similar dicyclic rhythms. The animals of these experiments were of comparable age and body weight (150 gms) at the time of each analysis, and had been housed for at least two weeks in constant temperature (70° F.) rooms fully exposed to natural lighting. Standard rat feeding cubes and water were supplied freely. Blood samples were taken at the same time of day on each occasion. The range of variation; 85%–115% of the annual mean for serum cholesterol and 80–120% for fatty acids, is typical of the majority of the examples to be considered. Major peaks occur in the spring and autumn with minima in summer and winter. However, the timing of these and their magnitude varies to some extent in successive years.

In Fig. 1C the response of the serum cholesterol of the rat to standard treatment with the compound I.C.I. 28,257 (0·25% administered in the

diet for 2 weeks) is seen to vary synchronously with, but with a magnitude inversely proportional to, the control levels of serum cholesterol. Note that in this figure the scale of the ordinate is inverted to facilitate

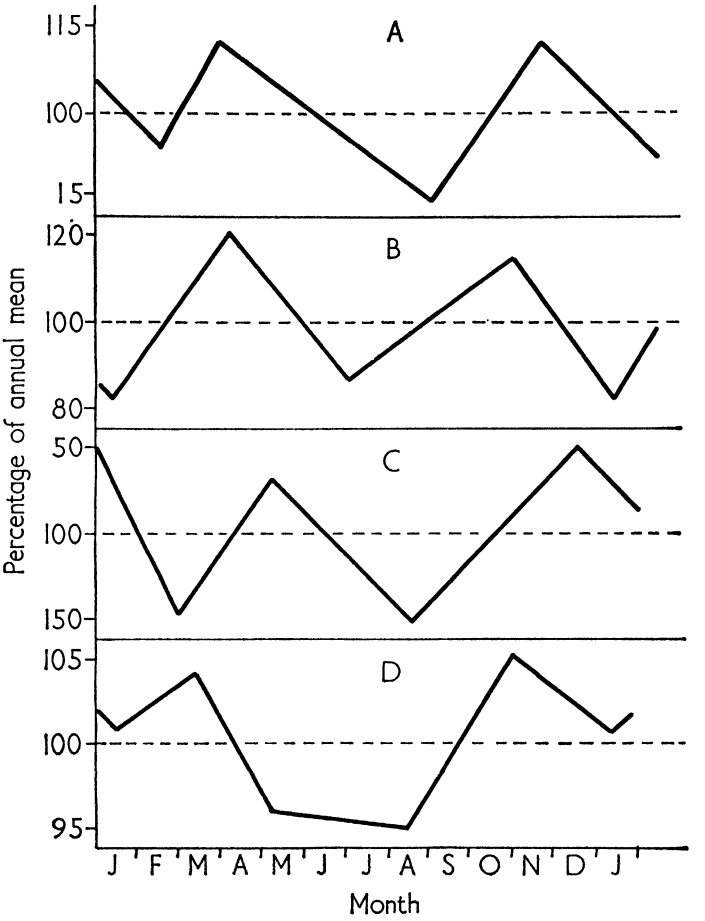


Fig. 1. Major patterns of variation of the blood lipids.
A: Serum cholesterol of rat: 100% equals 71·5 mg./100 ml. (Thorp & Waring, 1962.)
B: Plasma free fatty acid of rat: 100% equals 370 μEq./l. (A. M. Barrett, 1963, personal communication.)
C: Reduction of serum cholesterol of rats by standard treatment with I.C.I. 28,257; 100% equals 32% reduction. (Thorp & Waring, 1962.)
D: Serum cholesterol in man (police): 100% equals 232 mg./100 ml. (Paloheimo, 1961.)

comparison of this pattern with those of Figs. 1A and 1B. It is seen that the variation of response to treatment is roughly double the variation found in control animals. The nature of this response focused attention on the probable role of the endocrine system in determining the seasonal variation of serum lipids.

Figure 1D exemplifies the magnitude and nature of seasonal variations in serum cholesterol in man. The amplitude and frequency of this

variation differs according to location of the study and with the occupation, age and diet of the subjects studied. Because the seasonal pattern varies considerably between individuals, analysis of the average value for a group tends to obscure this pattern in proportion to the size of the group. If the annual variation of serum cholesterol of individuals is examined, a great majority show two peaks, which generally occur in the spring and autumn. We have analyzed the data of Paloheimo (1961), using a weighted moving average M, where:

$4M = 2 \times$ value for month of analysis $+$ sum of values for preceding and following months.

For one group of 44 subjects (police), the numbers showing 1, 2 or 3 maxima in serum cholesterol in the course of the year are 10, 31 and 3 respectively; and for the other group of 35 subjects (convicts), the numbers are 12, 20 and 3. Thus 65% of the seasonal patterns are dicyclic, and only 28% monocyclic. Yet when the results are averaged the predominant impression is of a monocyclic pattern, with a maximum in winter and a minimum in summer. But the minor cycle, from autumn to spring, shown in Fig. 1D, is undoubtedly real.

VARIATION IN ENDOCRINE FUNCTION IN THE RAT

The dicyclic annual variation of the serum cholesterol and plasma free fatty acid of the rat was found to bear a close resemblance to the variations in adrenal weight and adrenal ascorbic acid of control rats observed in an earlier series of experiments. These are shown in Fig. 2A and 2B, the latter illustrating two consecutive years' findings. At the two periods of relative hyperfunction (February/March and September/October) adrenal weight is increased and ascorbic acid content decreased. As these measurements were not made in the same years as those of the serum lipids, the temporal relationships between the variables are not clear.

The variation in mortality of rats exposed to a standard trauma (Noble–Collip drum test) is shown in Fig. 2C. This test measures mainly the protective influence of adrenocortical steroids. These results are somewhat out of phase with the indices of adrenocortical function of Figs. 2A and 2B. As minima may follow maxima within 1 to 1½ months, a displacement of the cycle by as little as one month may lead to this difficulty in correlating the time scale of various parameters. Figure 2D is a further example of a function probably largely under endocrine control, the "delayed" diuresis of rats following a saline load. The maxima in summer and in winter are notable, as this is contrary to what would be expected of a response dependent on temperature alone.

VARIATION IN ENDOCRINE FUNCTION IN MAN

Diurnal variation in adrenal and thyroid function in man is well recognized, but its interaction with lipid metabolism cannot be easily separated from other variables such as activity and feeding. As a measure of

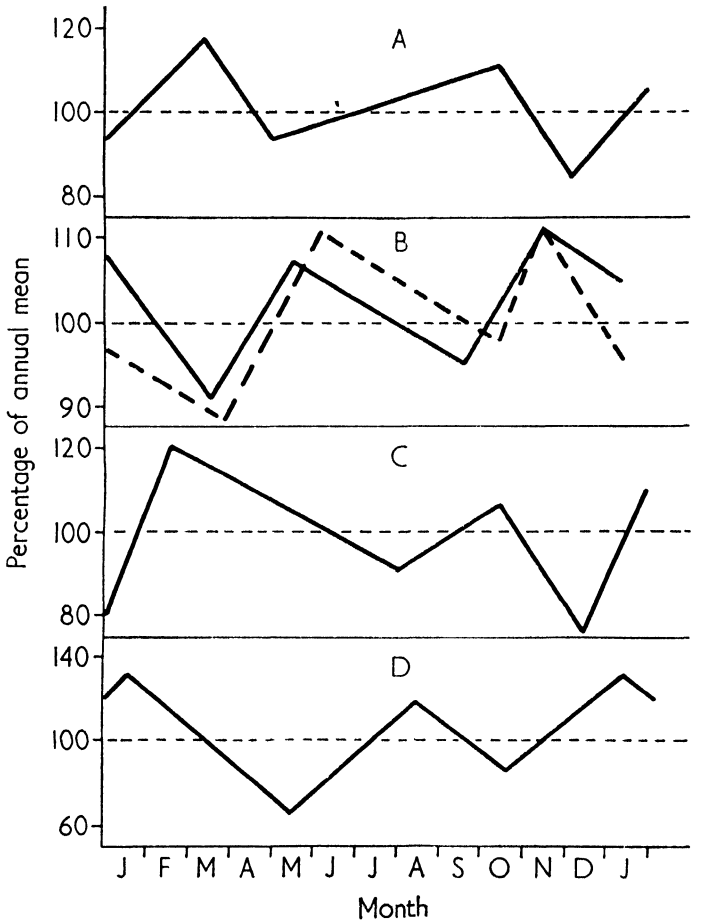


Fig. 2. Major patterns of seasonal variation of endocrine function in the rat as evidenced by:
A: Adrenal weight: 100% equals 9·65 mg./100 g. body weight. (Thorp, unpublished observations.)
B: Adrenal ascorbic acid: 100% equals 375 mg./100 g. fresh adrenal tissue. Results of two consecutive years shown separately. (Thorp, unpublished observations.)
C: Mortality of rats exposed to standard drum trauma: 100% equals 76% mortality. (Reichard, Gordon and Tesamer, 1960.)
D: Diuresis from 5 to 24 hours following a saline load: 100% equals 32% of dose. (A. Spinks, 1960, personal communication.)

thyroid function we have determined the time of the reflex contraction and relaxation of the Achilles tendon, at fortnightly intervals, in 31 normal men. This time interval, generally referred to (rather imprecisely), as the "reflex time" is regarded as a sensitive index of the state of thyroid function (Sherman, Goldberg & Larson, 1963). The results of our study are shown in Fig. 3A. As discussed in connection with serum cholesterol, the range of the variation of the average for the group is reduced to about one half of that of most individuals. Three maxima, corresponding to relative thyroid hypofunction, are found to

occur. The peak in mid-summer is unusual, and may be related to atypical weather conditions. Figure 3B illustrates a marked variation in plasma protein-bound iodine, which may reflect changes in the rate

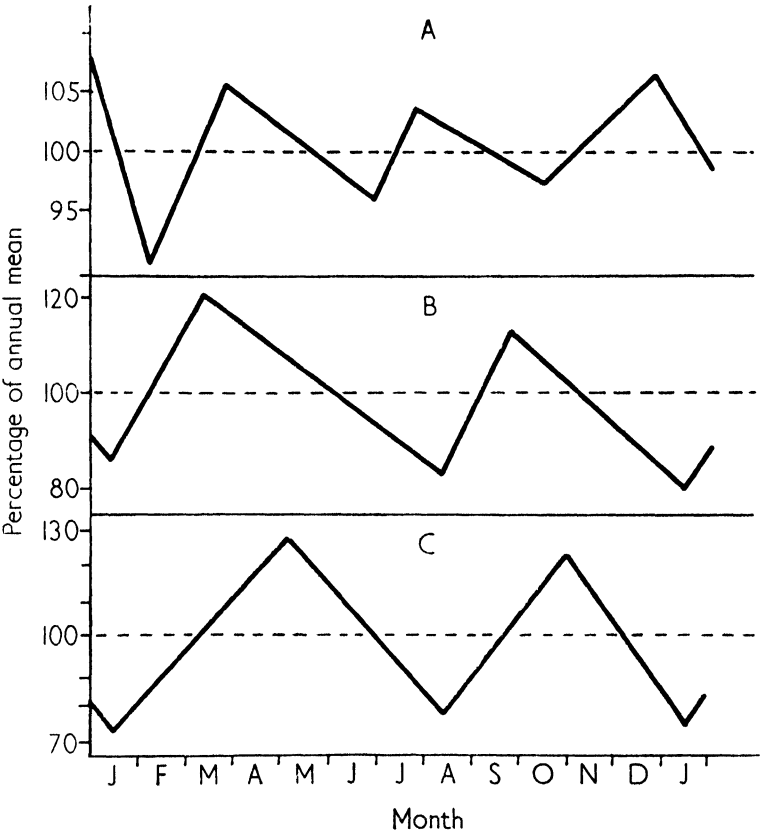


Fig. 3. Major patterns of seasonal variation of endocrine function in man.
A: Achilles tendon response. 100% equals 306 m.sec. (Thorp & Broad, 1963.)
B: Plasma protein-bound iodine: 100% equals 5·25 μg./100 ml. (Uematsu, 1961.)
C: Circulating eosinophils: 100% equals 175/cu.mm. (Watanabe, Aoki & Nagai, 1956.)

of utilization, or in the rate of secretion of thyroid hormone. In Fig. 3C a dicyclic variation of eosinophils in blood is shown. Fotherby, Strong & Thorp (1963) have confirmed the presence of this eosinophil variation, which was found to be inversely related to a variation in urinary excretion of 17 hydroxy-corticosteroids. Thus the spring and autumn maxima of eosinophil levels probably correspond with periods of relative adrenocortical hypofunction.

CONCLUSION

The correspondence between the seasonal variations of lipid levels in blood and of endocrine function reaffirms the interdependence of an animal with its environment and of its metabolism with its endocrine

system. The early experiments of Burn & Ling (1928) demonstrated marked changes in liver glycogen and in ketonuria in the rat, and in the response of these to insulin treatment, at different times of the year. More recently, Rubin & White (1959), describing the influence of hormones on lipid biosynthesis in liver had to separate this into two separate periods of study; and discontinued these studies because of the seasonal variable (A. White, 1960, personal communication). There is not only a seasonal difference in the basal state of lipid metabolism, but also in the extent to which it responds to external manipulation, hormonal or otherwise.

It is possible that these longer rhythms of function can serve to provide clues to the aetiology of the disturbances of lipid metabolism found in diabetes, obesity or atherosclerosis. Moreover, the endocrine rhythms, when more precisely analyzed, may prove to be of greater relevance to these long-term problems than are the results of "acute" variation in the level of circulating hormone or lipid metabolism.

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DISCUSSION

J. B. Weiss: We have seen exactly the same type of seasonal variation in the response of mice to injections of fat mobilizing peptides of the pituitary gland. This response was measured in terms of increases in liver fat and was very low in spring and autumn. It is interesting that two peaks also occur in the plotted values for liver fat in the untreated control animals, but these do not correlate with the variations in sensitivity of the animals to the injected peptides. These findings have been consistent and have been obtained over a period of more than three years. We find that in spring and autumn there is a lessening in sensitivity to the peptides and that in *late winter* (I stress the difference from spring), and autumn there are high liver fat values for the control animals. As in late winter the high control values are from groups of animals which also show the *highest* sensitivity to the peptides, there seems to be no doubt as to the lack of correlation of these two findings.

J. M. Thorp: These are very interesting observations, and confirm the difficulty of correlating differing seasonal changes. Do your findings apply to animals examined simultaneously, or were they obtained in different years?

J. B. Weiss: Control and treated animals were examined simultaneously over the three-year period.

P. H. Wright: We have seen the same type of seasonal variation in glucose uptake by rat diaphragms in the years 1955–1958. The glucose uptake showed peaks in the middle of Winter and in May–June.

R. A. Morton: Would you expect to have a minimal variation at the equator?

J. M. Thorp: Yes, I think that at the equator there would be little seasonal variation, also perhaps at the poles, since the rate of variation of the length of day appears to correlate best with the seasonal pattern. In animals maintained from birth in our breeding unit under constant environmental conditions, including regular light-dark cycles, we have found no seasonal variation of serum lipids. It is interesting that one can correlate serum cholesterol in man just as well with the latitude at which the observations are made as with such other variables as dietary fat intake.

PHARMACOLOGICAL CONTROL OF LIPID MOBILIZATION AND TRANSPORT

By RODOLFO PAOLETTI

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Many drugs have been found to be able to induce an increased outflow of free fatty acids (FFA) from the fat depôts and fatty deposition in the liver of normal animals.

Most of these drugs have in common the ability to stimulate the pituitary-adrenocortical axis. The increased levels of plasma corticosteroid induce an increased release of albumin-bound plasma free fatty acids and the excess of these is accumulated in the liver as triglycerides.

In other cases drug-induced fatty liver has a different origin, because it does not follow an increase of plasma free fatty acids but is related to a decrease of plasma triglycerides. This suggests a direct effect of the drugs on the liver, with impairment of the physiological mechanism regulating triglyceride secretion.

The present data show that the pituitary-adrenocortical axis and the peripheral sympathetic nervous system are important factors in liver triglyceride deposition produced by drugs modifying fatty acid mobilization. They also show that the hormones of the adrenal cortex may interfere with fat deposition in the liver after treatment with drugs directly acting on the liver secretory mechanisms.

THE RÔLE OF THE PITUITARY-ADRENAL AXIS AND SYMPATHETIC NERVOUS SYSTEM IN LIPID MOBILIZATION

Drugs and conditions such as exposure to cold, which cause ACTH release or exogenous ACTH itself induce increased plasma free fatty acid and fatty liver in normal animals, but not when the pituitary-adrenal axis is surgically inactivated (Paoletti, Maickel, Smith & Brodie, 1963).

Morphine is a drug able to induce fatty liver through this mechanism, particularly studied in this laboratory. The effect of morphine on the adrenal ascorbic acid levels, on the tryptophanpyrrolase activity in the liver and on the plasma corticosterone levels have been measured using different doses and times (Vertua & Usardi, 1962). The conditions of maximal adrenal stimulation have been identified and the effects on

lipid metabolism investigated. The results show a strict relation between the adrenal stimulation, the increased mobilization of fatty acids from adipose tissue and the induction of fatty liver (Vertua & Usardi, 1962).

The discovery of high concentrations of noradrenaline in adipose tissue, and the effects observed after its depletion with reserpine (Paoletti, Smith, Maickel & Brodie, 1961) together with indirect evidence from investigations carried out in different laboratories, suggest a permissive rôle for hormones in the increased release of free fatty acids, when the pituitary adrenal axis is stimulated.

Our present research has been focused on the rôle of the sympathetic nervous system after ACTH administration or exposure to cold. A peripheral chemical sympathectomy may be obtained with reserpine or guanethidine treatment in adrenal-medullectomized rats. The results expressed in Table 1 clearly show that an increased energy supply in

Table 1. *Chemical sympathectomy and lipid transport*

Treatment	Groups	Body temperature °C	Plasma Free Fatty Acids μEq/ml	Liver Triglycerides mg/g Tissue
—	Normal	(8) 39 ± 0·22	(14) 0·35 ± 0·03	(14) 4·8 ± 0·40
ACTH	Normal	—	(6) 0·66 ± 0·04	(6) 11·6 ± 1·00
(200 IU/Kg s.c.)			(6) 0·30 ± 0·03	(6) 5·2 ± 0·60
,,	C.S.*	—		
,,	C.S. + NA†	—	(6) 0·48 ± 0·04	(6) 8·5 ± 1·00
Cold exposure				
(2 hours)	Normal	(8) 37 ± 0·51	(8) 0·78 ± 0·06	(8) 8·5 ± 1·20
,,	C.S.*	(8) 22 ± 1·28	(8) 0·30 ± 0·04	(8) 4·2 ± 0·60
,,	C.S. + NA†	(8) 34 ± 0·85	(8) 0·58 ± 0·06	(8) 6·8 ± 0·70

Number of animals in parentheses.
* Chemical sympathectomy: adrenal medullectomy 25 days before the experiment, and treatment with guanethidine (2 doses of 15 mg/Kg each, 20 hours and 2 hours before the experiment).
† Administration of noradrenaline (1·5 mg/Kg s.c. in olive oil) at the beginning of the experiment, 2 hours before death of the animal.
Plasma Free Fatty Acids titrated according to Dole (1956); liver and plasma triglycerides measured according to Blankenhorn & Rouser (1961). Rats: male Sprague-Dawley, average weight 180 ± 10 g, fasted for 16 hours before CCl_4 administration.

the form of free fatty acids after adrenal stimulation can be only obtained when catecholamines are available. In absence of sympathetic tonus the animals, in a cold environment, cannot regulate the body temperature through release of excess energy, but this effect may be almost completely antagonized by administration of noradrenaline.

AN "IN SITU" TEST FOR DIRECT EVALUATION OF FREE FATTY ACID MOBILIZATION

The evaluation of data obtained from intact animals is fairly complicated because catecholamine depletion and administration may induce several side effects. A more direct tool to investigate the effects of the sympathetic transmitter on free fatty acid release is therefore highly

desirable. A new *in situ* perfusion test has been designed for this purpose, using the omental fat in dogs.

The procedure is as follows. Male dogs, fasted for 6 hours before the experiment, are anesthetized with an intravenous injection of sodium pentobarbital (25 mg/kg.). The animal is heparinized, kept under controlled respiration and body temperature during the experiment, and the haematocrit values repeatedly measured. The abdominal wall is opened along the xiphopubic line for two-thirds of its length. The omental vessels are separated from the general abdominal circulation, an artery and a vein are respectively connected with a carotid artery and a jugular vein using plastic tubes complete with side arms for administration of drugs or removal of blood samples. The spleen is removed in order to avoid a massive release of red blood cells, after catecholamine injection.

Under such conditions the omental fat releases only small amounts of free fatty acids (no significant differences between the arterial and venous concentrations), but the local administration of small doses of noradrenaline (about 0·1 μg) induces a sharp increase of free fatty acids in the plasma of the efferent vein (Table 2).

Table 2. *The* in situ *effect of noradrenaline on free fatty acid release from dog omental fat*

Treatment	Plasma Free Fatty Acids† μEq/ml	Plasma flow ml/min.	Plasma Free Fatty acids μEq/min.
None	0·49	3·44	1·68
Noradrenaline*	0·89	3·23	2·91

* Noradrenaline (0·1 μg) injected *in situ* intraarterially.
† Plasma free fatty acids measured 10 min. after noradrenaline administration.

This increase does not depend on a change in the blood flow because it is also seen with doses of noradrenaline unable to modify the volume of blood leaving the omental fat in the time unit (Table 2). On the other hand, doses of pitressin able to reduce considerably the blood flow do not modify the free fatty acid output.

An additional demonstration of the ability of this test to give direct data on the rôle of sympathetic innervation on free fatty acid release is obtained using an agent like nicotine, well known to increase blood free fatty acids even at small doses (Havel, 1963; Kershbaum, Bellet, Caplan & Feinberg, 1961; Kershbaum, Bellet, Caplan & Feinberg, 1962).

Nicotine has been administered in scalar doses in the afferent artery and the free fatty acids in the efferent venous blood have been measured at 4 and 8 minutes (Table 3). An increase in free fatty acid release is observed after a dose as low as 6·25 μg of nicotine. The effect is already maximal after 4 minutes, but it is still evident after 8 minutes. A larger

Table 3. In situ *effect of nicotine on free fatty acid release from dog omental fat*

Treatment	Plasma Free Fatty Acids after 4 minutes μEq/ml	after 4 minutes μEq/min.	after 8 minutes μEq/ml	after 8 minutes μEq/min.
None	0·30	1·25	0·30	1·25
Nicotine* 6·25 μg	0·51	3·21	0·43	3·32
Nicotine 12·5 μg	0·56	1·79	0·52	1·82

* Nicotine bitartrate, administered *in situ* intraarterially;
Plasma Free Fatty Acids (FFA) collected from the effluent vein, 4 and 8 minutes after the nicotine administration.
The value μEq/min. of FFA indicates the total output (μEq FFA/ml. plasma) × (ml. plasma/minute).

dose (12·5 μg) prolongs the maximal output over a period of at least 8 minutes.

The active doses on this test are much lower than the active dose in the intact animal and this suggests the possibility of using this test in many problems concerning lipid mobilization.

THE REGULATION OF LIVER TRIGLYCERIDE SECRETION INTO THE PLASMA

The oral administration of CCl_4 induces an important and rapid increase of liver triglyceride concentration and a simultaneous decrease of plasma triglycerides (Table 4). These results, together with the unchanged level of plasma free fatty acids, are in sharp contrast with the

Table 4. *Effects of CCl_4 administration on lipid transport*

Treatment	Plasma Free Fatty Acids	Liver Triglycerides	Plasma Triglycerides
None	100	100	100
Oral CCl_4 (2 hours)	98	149	64
Oral CCl_4 (4 hours)	83	302	36
Oral CCl_4 (8 hours)	—	387	37

CCl_4 (5 ml/Kg 1:1, v/v in mineral oil) by gastric intubation at 2, 4, 8 hours before the animal is killed. The data represent the average of 12 animals.

data observed after administration of ACTH or ACTH-releasing drugs and suggest a site of action at the liver level for CCl_4. Different possible mechanisms have been proposed in recent years.

Lombardi & Recknagel (1961) suggest an action for CCl_4 on the liver triglyceride secretory mechanism, because in CCl_4-treated rats Triton-induced hyperlipemia is considerably reduced.

Calvert & Brody (1960) observed that lipid infiltration in the liver may be decreased or prevented by a section of the spinal cord, adrenalectomy or administration of adrenolytic drugs. Fatty infiltration is related to a centrilobular necrosis, which is also reduced by such treatments. According to these authors, CCl_4 should act centrally by causing a massive discharge of catecholamines. The excessive amount of released neurohormones are believed to produce vasoconstriction and the

centrilobular lesions. This interpretation, however, is not in agreement with the observation that plasma free fatty acids are not elevated after oral CCl_4 administration, but liver triglycerides are increased. In our experimental conditions, the catecholamines of the adrenal medulla seem to play a minor rôle in fatty liver induced by CCl_4.

Adrenal medullectomized rats show an almost normal increase of liver triglycerides after carbon tetrachloride (Table 5). Preliminary

Table 5. *Effect of adrenal medullectomy on CCl_4 induced modification of lipid transport*

Groups	Animals	Treatment	Liver Triglycerides mg/g (±S.E.)	Plasma Triglycerides mg/100 ml (±S.E.)
I	Normal	None	(5) 3·60 ± 0·22	(5) 61·0 ± 3·5
II	Normal	CCl_4	(5) 8·49 ± 0·36	(5) 27·8 ± 3·9
III	Medullectomized	None	(5) 4·10 ± 0·47	(5) 68·1 ± 7·9
IV	Medullectomized	CCl_4	(5) 8·19 ± 0·42	(5) 44·6 ± 10·3

Bilateral adrenal medullectomy performed 25 days before the experiments; CCl_4 (5 ml/Kg, 1:1, v/v, in mineral oil) given by gastric intubation 4 hours before the death of the animal. Number of animals in parentheses.
Significance levels:

Liver	I–II	$p < 0·01$	Plasma	I–II	$p < 0·01$
	III–IV	$p < 0·01$		III–IV	$p > 0·05$
	II–IV	$p > 0·05$		II–IV	$p > 0·05$

results obtained in rats after surgical removal of the adrenal medulla and treatment with BW329C60 (*ortho*-chloro-N-benzyl-N^1-N^{11}-dimethyl-guanidine sulphate, Burroughs Wellcome Laboratories) (Kuntzman, Costa, Gessa & Brodie, 1962), a powerful adrenergic-neurone blocking agent, suggest that the catecholamines have a modest rôle in this type of fatty liver.

The results are quite different when CCl_4 is administered to adrenalectomized animals. The fatty liver is not seen 2 and 4 hours after the treatment (Table 6) and the typical decrease of plasma triglyceride

Table 6. *The rôle of the adrenal cortex on the liver triglyceride levels after CCl_4 administration*

Groups	Animal	Treatment	Liver Triglycerides mg/g ± S.E. 2 hours after CCl_4	4 hours
I	Normal	None	(6) 4·4 ± 0·41	(8) 4·7 ± 0·51
II	Normal	CCl_4	(6) 6·5 ± 0·57	(7) 12·1 ± 1·38
III	Adrenalectomized	None	(6) 3·5 ± 0·15	(10) 4·1 ± 0·42
IV	Adrenalectomized	CCl_4	(6) 3·8 ± 0·38	(9) 6·1 ± 0·70

Adrenalectomy performed 72 hours before the experiment.
CCl_4 (5 ml/Kg 1:1, v/v, in mineral oil) given by gastric intubation at the hours specified before the death of the animal, number of animals in parentheses.
Significance levels:

2 hours	I–II	$p = 0·01$	4 hours	I–II	$p < 0·01$
	III–IV	$p > 0·05$		III–IV	$p > 0·05$
	II–IV	$p = 0·02$		II–IV	$p < 0·01$

concentration is cancelled (Table 7). This indicates that the adrenocortical hormones may play a permissive rôle on the secretory mechanism of triglycerides and that carbon tetrachloride should act at the level of such a regulatory physiological mechanism.

Table 7. *Plasma triglyceride levels after CCl_4 in adrenalectomized rats*

Groups	Animal	Treatment	Plasma Triglycerides mg/100 ml ± S.E.
I	Adrenalectomized	None	(8) 49·4 ± 3·80
II	Adrenalectomized	CCl_4 2 hours	(8) 42·2 ± 3·70
III	Adrenalectomized	None	(8) 33·4 ± 2·40
IV	Adrenalectomized	CCl_4 4 hours	(7) 35·1 ± 5·20

Adrenalectomy performed 72 hours before the experiments.
CCl_4 (5 ml/Kg, 1:1, v/v, in mineral oil) given by gastric intubation at the times specified, number of animals in parentheses.
Significance levels:
I–II = $p > 0{\cdot}05$
III–IV = $p > 0{\cdot}05$

This effect is not related to an increased lipid mobilization from the fat depôts, because under the selected conditions there is no important increase of plasma free fatty acids and the corticoids seem a permissive factor in the liver for the CCl_4-induced blockade of triglyceride secretion.

In order to verify this possibility, the effect of aldosterone and corticosterone treatment on the CCl_4 action on lipid transport has been tested. Aldosterone has no obvious effect in adrenalectomized animals, but corticosterone reverses the protective effect of adrenalectomy against CCl_4-induced lipid infiltration (Table 8).

Table 8. *Effect of corticosterone treatment on CCl_4 induced modification of lipid transport in adrenalectomized rats*

Groups	Treatment	Liver Triglycerides mg/g ± S.E.	Plasma Triglycerides mg/100 ml ± S.E.
I	Corticosterone	(12) 4·62 ± 0·28	(12) 32·3 ± 1·7
II	Corticosterone + CCl_4	(12) 11·32 ± 0·80	(12) 23·4 ± 1·0

Adrenalectomy performed 72 hours before the experiments.
CCl_4 (5 ml/Kg of 1:1, v/v, in mineral oil) given by gastric intubation and corticosterone (10 mg/Kg, s.c.) both 4 hours before the death of the animal, number of animals in parentheses.
Significance levels:
Liver I–II $p < 0{\cdot}01$
Plasma I–II $p < 0{\cdot}01$

This effect of corticosterone is not related to an increased lipid mobilization, because under the conditions of our experiment no significant increase of plasma free fatty acid has been observed.

The protection against liver fatty infiltration in adrenalectomized animals and its removal after corticosterone administration are certainly

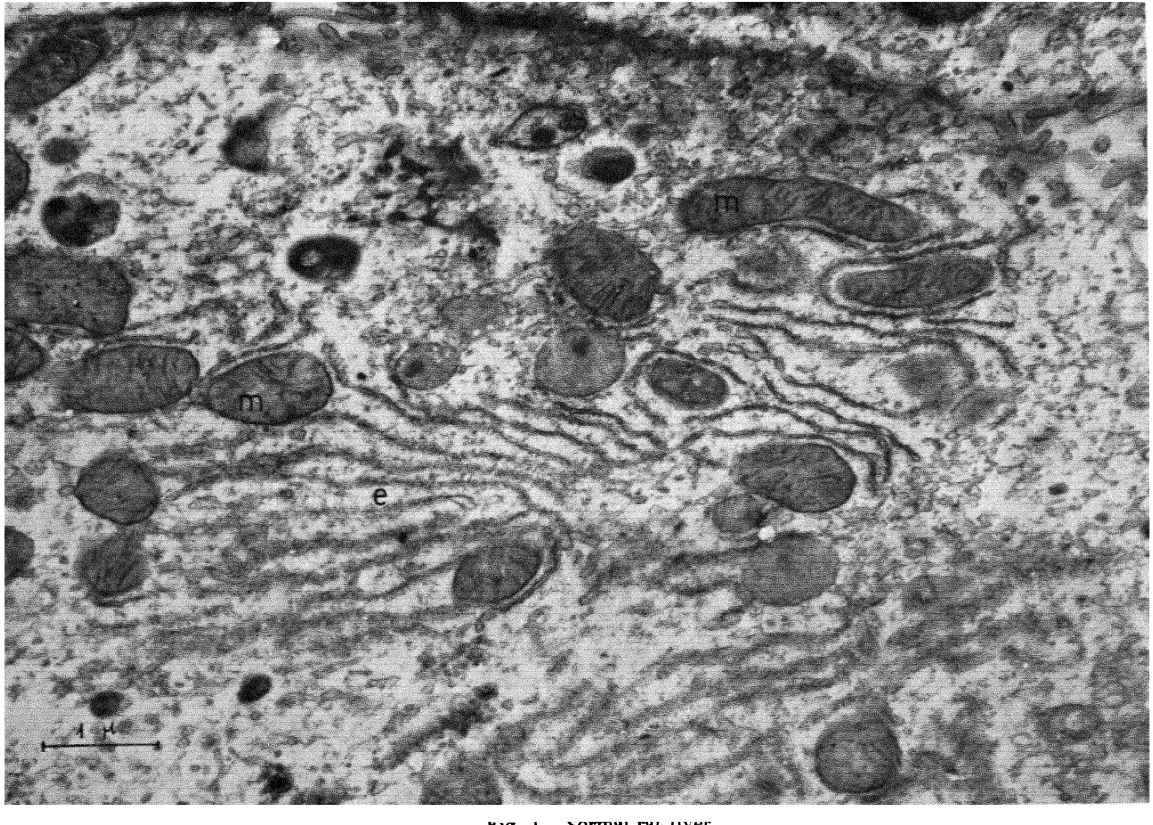


Fig. 1 Normal rat liver

Male Sprague-Dauley rats weighing 180 ± 10 g fasted for 20 hr. Liver samples fixed in osmic tetraoxide in Milloning buffer, embedded in Westopal. m = mitochondria. e = endoplasmic reticulum.

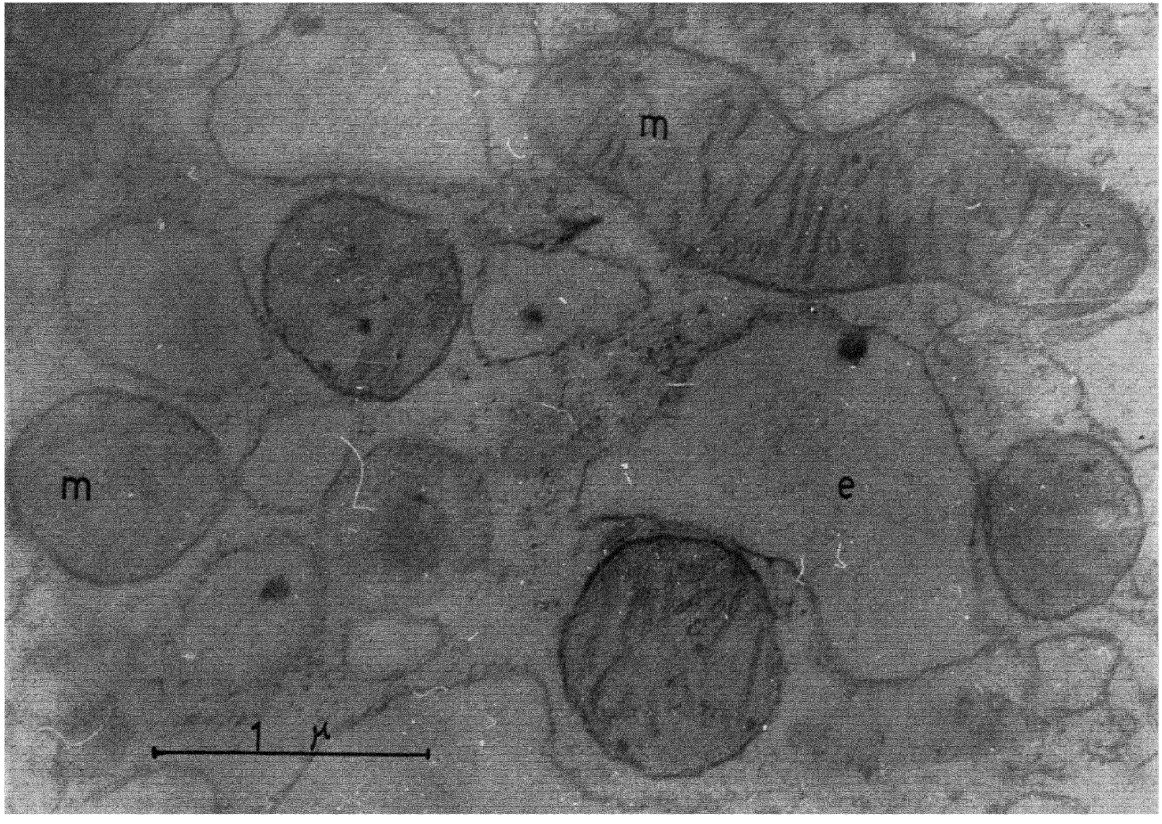


Fig. 2 Rat liver after carbon tetrachloride treatment

Rats treated with 5 ml CCP_4 (1:1, v:v in mineral oil)/kg 4 hr. before killing. Note enlargement of endoplasmic reticulum in contrast with normal mitochondria.

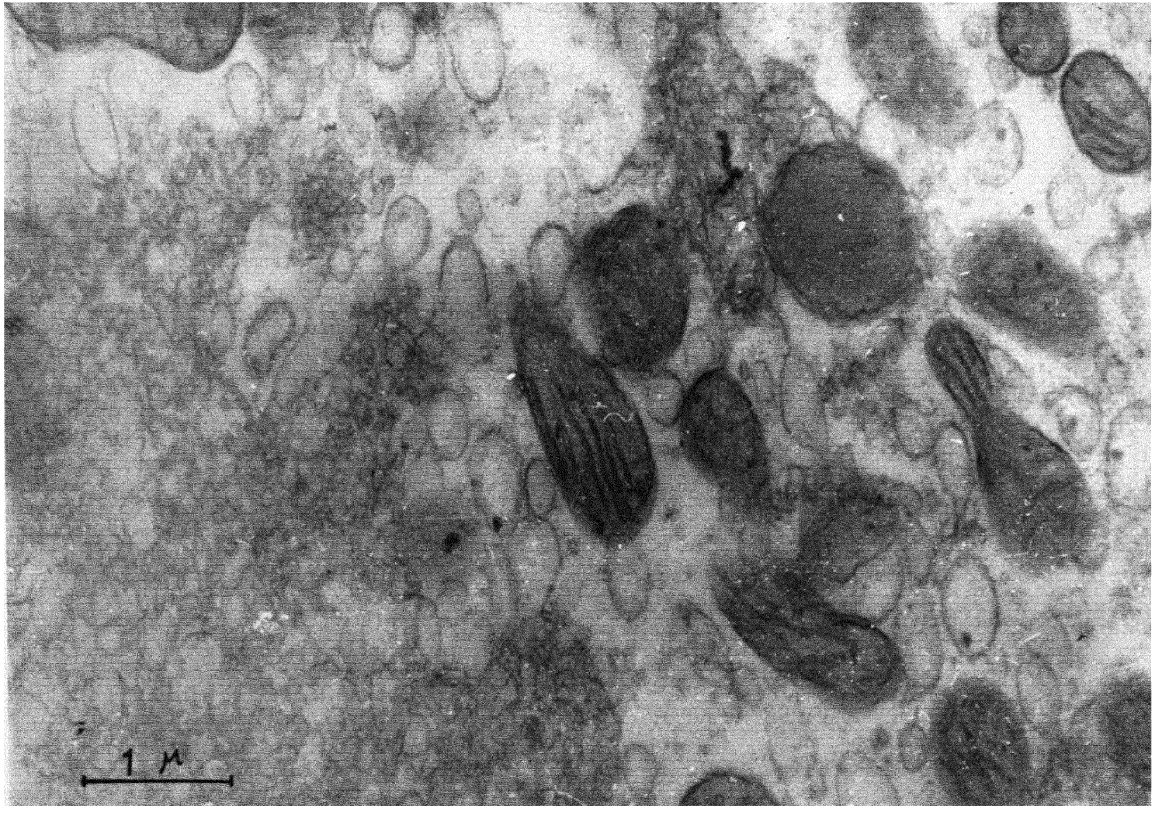


Fig. 3. Rat liver after carbon tetrachloride treatment
Same conditions as in Fig. 2. Evident alteration in endoplasmic reticulum with agglomeration of smooth membrane vesicles.



Fig. 4. Adrenalectomized, **untreated** rat liver
Rats **submitted** to bilateral **adrenal**ectomy 48 hr. before **killing.** No alteration of the normal **structures.**

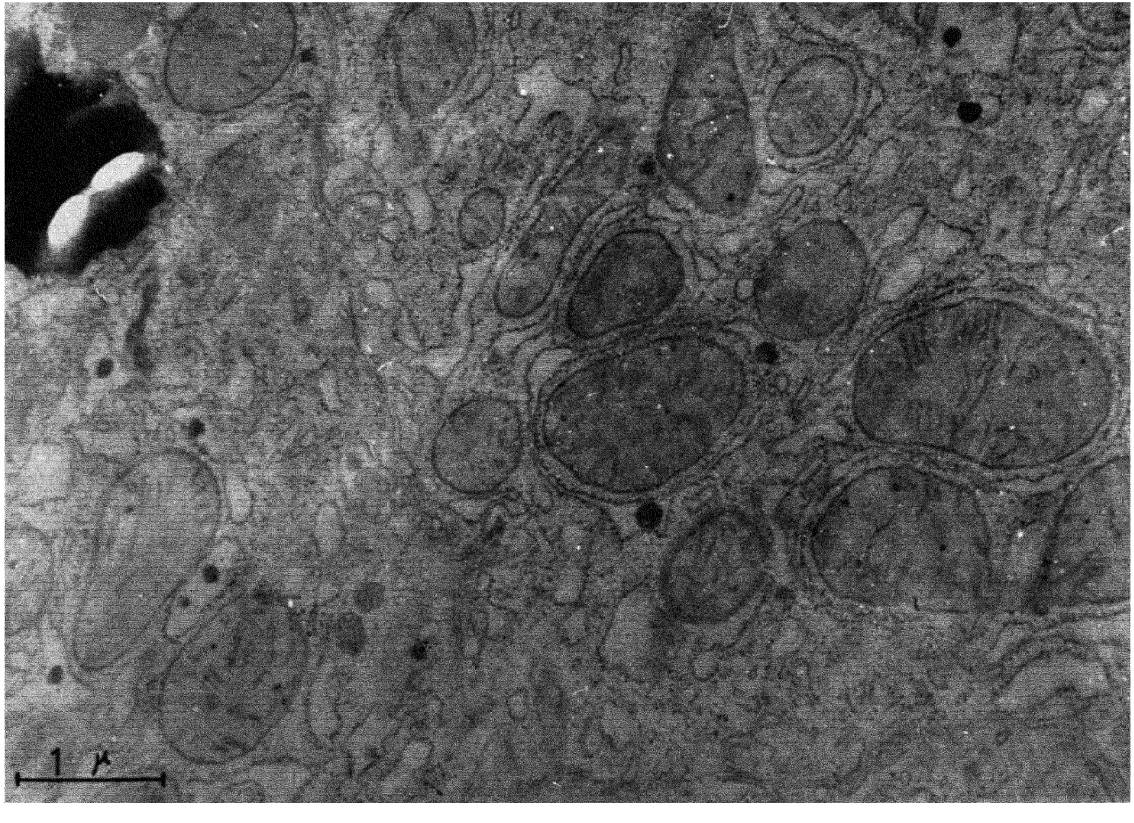


Fig. 5. Liver of carbon tetrachloride-treated adrenalectomized rats
Experimental conditions as in Fig. 2; using adrenalectomized rats. Modest and scattered dilatation of the endoplasmic reticulum.



Fig. 6. Liver of carbon tetrachloride-treated adrenalectomized rats given corticosterone
Damage of the endoplasmic reticulum comparable to that observed in Figs. 2 and 3. An agglomeration of smooth membrane vesicles is present (as in Fig. 3).

related to more fundamental modifications of the liver cell response to carbon tetrachloride intoxication.

In normal rats, after oral CCl_4 treatment, an early and constant increase of some serum enzymes may be observed. Glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase levels are doubled 4 hours after CCl_4 administration and the values of lactic dehydrogenase are three times the normal figures 8 hours after CCl_4. The earliest increase is shown by the glutamic-pyruvic transaminase activity in the serum (44% increase after 2 hours), indicating a specific alteration of the liver tissue (Table 9). In adrenalectomized animals glutamic-pyruvic transaminase is unchanged and the other two enzymes only slightly increased.

Table 9. *Serum enzymes after CCl_4 administration in normal and adrenalectomized rats*

Animals	Treatment	LDH	SGOT	SGPT
Normal	None	100	100	100
Normal	Oral CCl_4 (2 hours)	89	128	144
Normal	Oral CCl_4 (4 hours)	124	239	267
Normal	Oral CCl_4 (8 hours)	319	733	290
Adrenalectomized	None	100	100	100
Adrenalectomized	Oral CCl_4 (2 hours)	104	104	102
Adrenalectomized	Oral CCl_4 (4 hours)	128	131	96

CCl_4 (5 ml/Kg, 1:1, v/v, in oil) administered by gastric intubation, at the hours specified, before the death of the animal.
Each figure represents the average of 12 animals.
Normal values: LDH 1538·0 U/ml, SGOT 77·8 U/ml, SGPT 30·6 U/ml (averages for 25 animals).
Lactic dehydrogenase (LDH), glutamic-oxalacetic transaminase (SGOT) and glutamic-pyruvic transaminase (SGPT) are measured respectively according to the methods described by Wroblewski & LaDue (1955), Karmer, Wroblewski & LaDue (1955), and Wroblewski & LaDue (1956).

The alterations of the liver cells in the conditions considered are clearly demonstrated by the electron microscope (Figs. 1–6). The damage, specific for the endoplasmic reticulum, is very pronounced 4 hours after carbon tetrachloride administration, in the normal but not in adrenalectomized animals. After corticosterone treatment the alterations are almost the same as in normal animal, in good agreement with the chemical data.

CONCLUSIONS

The use of pharmacological agents active at different levels may give useful information on the physiological mechanisms involved in the regulation of lipid mobilization, transport and deposition.

The free fatty acid release from adipose tissue is under the combined control of corticosteroids and catecholamines and even an excess of one of these agents induces no stimulation unless a certain concentration of the other is present in the system.

The rate of triglyceride secretion from the liver into the plasma—a process which regulates liver and plasma triglyceride levels—seems under the control of corticosteroid. The chemical and morphological alterations in liver cells observed after CCl_4 administration in normal, but not in adrenalectomized animals, are a clear-cut example of the sequence of events accompanying the derangement of lipid transport control.

Further research along these lines will facilitate the understanding of some of these regulatory mechanisms and it will possibly give a more accurate model of energy supply in normal and emergency conditions.

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DISCUSSION

H. B. Stoner: Do you know the concentration of carbon tetrachloride in the liver of the rats which have been adrenalectomized? It is important in these cases to be able to show that the lesion could have occurred.

R. Paoletti: It does not differ from the amount found in the livers of intact animals treated with carbon tetrachloride. As you have probably noticed the dose of carbon tetrachloride is unusually large. We used this dose in order to have a quick effect on lipid transport before any serious mitochondrial lesion occurs, and these normally occur very quickly. There is usually the same amount of carbon tetrachloride 1 hour after the oral administration, provided that the animals are kept at 37°C.

D. S. Robinson: With respect to the agents causing fatty liver due to excessive fat mobilization, we have been puzzled as to why the plasma triglyceride level should rise as dramatically as it does. One explanation is that the disposal of plasma triglyceride by the tissues is inhibited. Clearing factor lipase is decreased in the tissues but only at an early period, usually by about 6 hours, whereas the maximum triglyceride level was found at a later stage. This question of the reason for the high plasma triglyceride level has not yet been fully explained. I do not think our results are adequate to explain it.

Another point relates to the carbon tetrachloride fatty liver. The observed decreased triglyceride release from the liver may be accounted for by reduced synthesis of the plasma lipoprotein moiety which seems to occur with carbon tetrachloride, white phosphorous, and methionine, although not necessarily by the same mechanism. This is observed with ethanol. I wonder if you are justified in putting ethanol in your first group?

R. Paoletti: As regards your first point, it is interesting to observe that after hypermobilization of free fatty acid induced by drugs, a long time, 12 hours at least, elapses before there is a hyperlipaemia, even with large doses of drugs. I do not think there is any good explanation for this delay. Methionine and carbon tetrachloride have quite different effects on the liver. After methionine treatment cholesterol levels in blood are decreased and after carbon tetrachloride they are normal. I am not quite happy about the classification of the alcohol effects. Possibly alcohol is a more complicated agent as far as concerned lipid transport than we generally think. Certainly I agree that plasma protein synthesis plays an important rôle in the fatty liver induced by carbon tetrachloride.

D. S. Robinson: We find the decreased cholesterol levels in carbon tetrachloride treated animals. We observe this both with methionine and carbon tetrachloride treated animals.

R. Paoletti: A much more pronounced fall occurs after methionine than after carbon tetrachloride. In adrenalectomized animals there is still a decrease in cholesterol levels in blood after methionine. I believe these effects are quite different.

E. Assem: Is it possible that these drugs by their effect on corticosteroid production would effect liver and this would in turn influence the "inactivation" of corticosteroid?

R. Paoletti: We do not have data on this point.

A. M. Barrett: We have found that the administration of a large dose of adrenaline, 2 mg/kg subcutaneously, gives an enormous rise in fatty acids in a fed rat. When the animal is examined 24 hours later we do not

find any increase in plasma triglycerides. We have measured plasma triglyceride by determining the total esterified fatty acid and then subtracting from it the cholesterol and phospholipid fractions, determined separately. That is by a difference method. Do you think our failure to find any difference in plasma triglycerides is due (a) to a strain difference in the rats, (b) to the method of determination that I am employing or (c) is it in any way connected with nutritional status of the rat at the time at which the fatty acid mobilizing hormone is given?

R. Paoletti: I think a possible reason is wrong selection of time. For instance, after two days of treatment with ACTH one gets fatty livers. The increase in blood triglyceride occurs later.

A. N. Howard: We found that the estimation of plasma triglyceride by the difference method was in fact quite useless particularly in the rat. We obtained negative values and we found that the only solution was to make the determination directly and I gather that Professor Paoletti does in fact use this method.

A. M. Barrett: Initially we used this difference method with a dog. At this time we were using an auto analyser to determine the total esterified fatty acids. Since I have abandoned the auto analyser and measure free fatty acids and the esterified fatty acids, by hand as it were, I have never had negative results. I am quite happy when there, were no plasma triglycerides, but, like you, when I obtained large negative values I was rather puzzled as to how to interpret the result.

CONCLUDING REMARKS

By S. J. FOLLEY

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In closing this symposium it is instructive to recall the advances made since the previous symposium on lipid metabolism held by the Biochemical Society in 1952. Although that meeting was intended to review advances, recent at that time, in the biochemistry of lipids it has since transpired that it heralded a period of even more rapid progress in this field. Among important achievements since the previous meeting may be mentioned the elucidation of the malonyl CoA pathway of lipogenesis, the unravelling of the route of sterol biosynthesis through mevalonic acid, the recognition of the unesterified fatty acids of the blood as a major means of transport of energy-yielding substrates, the clarification of the mechanism of glyceride formation and the great advances in our understanding of the metabolism of adipose tissue.

It is significant that because of this and much other work it was possible to choose as the theme of today's meeting the control of lipid metabolism. This has been discussed in relation to various phases of this process, in organisms varying in complexity from bacteria to man, at different levels down to that of the individual enzyme. We have considered the possibility that acetyl CoA carboxylase might catalyse a rate limiting step in lipogenesis, the activity of which is modulated by citrate and its chemical relatives. Here we may be dealing with an allosteric transformation, a concept brilliantly propounded in a recent paper by Monod and his colleagues, although it may be noted that in this case the activator may also contribute carbon atoms to the substrate pool. The suggestion that higher acyl CoA derivatives might exert a "feed back" inhibition on the enzyme has also been made. We have as yet no unequivocal instance of a hormone directly affecting an enzyme system specifically involved in lipogenesis and although there are certain analogies between the hormonal activation of the lipase of adipose tissue concerned in the hydrolysis of triglycerides and of the phosphorylase present in various tissues it is probably premature to speculate on the possibility that this hormone-sensitive lipase is another example of an allosteric protein. If this should prove to be the case then the protein in question is singularly indiscriminating in its choice of hormonal activator.

Other control mechanisms which have been discussed include those

involving the availability of coenzymes such as NADPH and ATP, and in this connection one question which must interest us and remains unresolved is the relationship between the pentose phosphate pathway and lipogenesis. The importance of this metabolic pathway as a regulator of lipogenesis through the supply of NADPH has been questioned at this meeting. While it must be admitted that here we seem to be confronted by the old problem of the hen and the egg in that it is not certain whether the demands of a sudden increase in lipogenesis, as in the mammary gland at parturition, evoke a corresponding enhancement of pentose phosphate cycle activity or *vice versa*, the close coupling between these two metabolic processes cannot be disputed and the relationship between carbohydrate breakdown and lipogenesis remains a fruitful field for further research in which many puzzles have still to be solved. One example is the observation that in slices of lactating mammary tissue from ruminants which actively incorporate acetate into fatty acids, the addition of glucose to the medium markedly increases lipogenesis from acetate although glucose carbon scarcely contributes to the acetyl CoA pool, nor does insulin exert any stimulating effect as it does with rat mammary gland slices in similar circumstances. It is in the solution of problems of this sort that the lactating mammary gland, in which lipogenic activity varies markedly in different physiological states and to some extent between species, may be expected to prove a particularly useful tool. This tissue which is a target organ for many hormones and which exhibits an important lipid metabolism has hardly yet been adequately exploited by biochemists. Further studies on mammary gland slices or organ cultures as well as on adipose tissue may well throw light on hormone enzyme relationships.

At this meeting we have discussed many examples of the direct effects of hormones on lipid metabolism in isolated organs *in vitro* and we have also considered hormonal regulation of lipid metabolism, in its various aspects, at the level of the whole animal. In the interpretation of studies of the latter type the difficulty of separating primary endocrine effects from those resulting from secondary hormone adjustments needs no emphasis. In all studies involving anterior-pituitary protein hormones, whether *in vitro* or in the whole animal recent findings in respect of species specificity may necessitate some re-interpretation of existing data and must be taken into account in planning future work. Consideration of these endocrine studies suggests that there are two principal ways in which the nervous system and through it the environment participates in the control of lipid metabolism. The action of sympathecticomimetic amines in accelerating release of free fatty acids from adipose tissue stores illustrates the probable rôle of the autonomic nervous system. The other mechanism is mediated by the intervention of anterior-pituitary hormones, or the hormones secreted by their target organs, in various phases of lipid metabolism and involves the hypothalamic control of the secretory function of the pituitary gland.

The form of this meeting, with its emphasis on the discussion of pre-circulated papers, was an experiment as far as the Biochemical Society is concerned although this technique has long been successfully used by certain other societies. Today's discussion has been most stimulating and the success of this meeting may well point to the desirability of the Society's holding further discussion meetings in the future.

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