

# THE METABOLISM OF ACETIC ACID IN ANIMAL TISSUES

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Although acetic acid has long been suspected to be an intermediate in animal metabolism, the significance of its rôle in biochemical processes has only recently been recognized. The lack of interest in acetic acid metabolism during the past is in part attributable to the inadequacy of methods for identification and for quantitative determination of small amounts of acetic acid, and partly due to the fact that in isolated tissues acetic acid appears to possess only limited reactivity.

Until recently the principal approach to problems of intermediary metabolism has been the balance experiment, i.e., the measurement of the changes in concentration of the reaction product induced by varying concentrations of suspected precursor. The only reactions of acetic acid in animal tissues which could be reasonably well established by balance experiments were the acetylation of foreign amines (1, 2) and the formation of acetoacetic acid (3, 4, 5). A much greater variety of reactions involving acetic acid has been revealed with the aid of isotopically labeled substrates. The utility of the tracer technique for the study of intermediary metabolism derives from the fact that with its aid biochemical conversions become demonstrable irrespective of whether or not the total quantity of the reaction product undergoes a change. Thus the use of labeled acetic acid has been of particular service in detecting acetate formation and in demonstrating its participation in processes which proceed independently of exogenous acetate supply, viz., the biological syntheses of steroids, fatty acids, porphyrin, glucose and uric acid.

In general, it will prove more useful to review a broad segment of intermediary metabolism rather than the metabolism of a single compound. Indeed, the great diversity of biochemical processes, catabolic and anabolic, in which acetic acid is involved would require a much more comprehensive discussion of related subjects of intermediary metabolism than can be given here. Also, the present review is concerned mainly with the metabolism of acetic acid in animal tissues, although reference will be made frequently to processes occurring in other cells. This will be necessary because in many cases the discovery of a biochemical process in microorganisms has preceded and forecast an analogous reaction in animal tissues.

There is evidence to suggest that there exists a biologically active form of acetic acid but its identity has not been established. In the present review the terms acetic acid, acetyl and C<sub>2</sub> unit will be employed, sometimes interchangeably, without any intent of specifying a chemically defined entity.

**THE ACETYLATION REACTION.** The acetylation of foreign amines and amino acids will be discussed in some detail because this reaction has been of great value for the elucidation of various aspects of acetic acid metabolism in animal tissues.

Acetylation is one of the processes occurring with foreign amines prior to their elimination from the animal body. To what extent acetylation takes place in a given case will depend on the chemical nature and the dosage (6) of the foreign substance and on the species of the test animal. In rats, sulfanilamide is in part excreted unchanged, partly converted to N-acetyl sulfanilamide and oxidized to a hydroxyl compound which can subsequently be conjugated with glucuronic acid. No acetylation of aromatic amines such as sulfanilamide occurs in the dog (7, 13). Also, no unique mechanism exists for the disposal of foreign  $\alpha$ -amino acids of the type represented by  $\gamma$ -phenyl  $\alpha$ -aminobutyric acid or cyclohexyl alanine. Depending on experimental conditions, the feeding of these amino acids will yield varying proportions of N-acetyl amino acid, hydroxy acid, keto acid and oxidation products derived from the latter (8). Reactions of this kind have been classified as detoxications, a pharmacological term which is generally taken to mean that the body tends to modify foreign substances in such a manner as to render them less injurious to the cell either by increasing their solubility in biological fluids, thus preventing deposition of foreign bodies, or by conversion to derivatives which have less affinity for enzyme systems. The observation that in some instances the toxicity and insolubility of the conversion product exceeds that of the original compound, as in the case of acetyl-sulfanilamide (9), has made it evident that this type of change undergone by the foreign substance in animal tissues could no longer be adequately described as a detoxication (10, 11). Available evidence indicates that there are but few organic compounds which are entirely refractory to attack by animal cells. The fact that substances are metabolized which are not normally constituents of animal tissues does not necessarily imply the existence of a multitude of enzymes which operate only when the foreign substance is offered to the cell. Thus the coupling of glucuronic acid not only with camphor, borneol or phenol, but also with normally occurring steroid alcohols, or the acetylation of natural as well as of foreign amino acids suggests that these conjugation reactions are not induced by the administration of foreign substances but merely reflect events which are part of the normal intermediary metabolism. The acetylation of a foreign amine may then be attributed to an enzyme which has as its normal function the acetylation of a naturally occurring amine. The enzymes concerned in "detoxication" reactions have not been sufficiently characterized to answer the question whether there exist normal substrates for the enzymes which act upon foreign substances. The enzyme which catalyzes the acetylation of sulfanilamide has been purified (2), but the investigation of substrate specificity has not been extended to normally occurring amino acids. p-amino benzoic acid is acetylated by the same system although at a somewhat slower rate than sulfanilamide.

As an example of a purified enzyme which does not differentiate between a normal and foreign substrate, the l-amino acid oxidase of Green et al. (12) may be mentioned. In the presence of the enzyme, phenylaminobutyric acid, a foreign amino acid, is oxidatively deaminated more rapidly than any of the natural amino acids. Evidently, in this case enzymatic activity is determined

by the  $\alpha$  amino acid configuration and not by whether the amino acid is foreign to the cell.

The view that metabolic occurrences with foreign substances in animal tissues mirror normal events formed the basis for the classical investigations of Neubauer, Knoop and Dakin, who employed phenyl-substituted compounds as models for the study of fatty acids and amino acid metabolism. It attests to the validity of the original concepts that the conclusions arrived at by these investigators some forty years ago have not required any fundamental revision.

*Acetylation of amines.* Following the early finding of Cohn (13) that p-nitrobenzaldehyde was converted by the dog to acetyl p-aminobenzoic acid, acetylation was more closely investigated with the phenyl derivatives of glycine and of  $\alpha$ -aminobutyric acid (8, 14). Although these experiments were designed to study synthesis and degradation of amino acids, they yielded equally important evidence with regard to the participation of acetic acid in intermediary metabolism. Knoop (8) showed that the feeding of racemic phenylaminobutyric acid led to the excretion of an optically active acetyl derivative which he found to be identical with the acetyl compound formed in the animal from  $\gamma$ -phenyl  $\alpha$ -ketobutyric acid. Knoop erroneously assigned the unnatural configuration to this acetyl amino acid and concluded that the acid of natural configuration had been converted to the acetyl derivative of the unnatural isomer. Similarly the acetyl derivative of what Knoop believed to be l-phenylalanine was excreted unchanged while the acetyl derivative of the amino acid of opposite configuration, judged from the behavior of the racemic acetyl amino acid, appeared to be readily metabolized (15). In view of these findings Knoop was unable to retain his original suggestion that acetylation was a process of normal amino acid metabolism. However, when the configuration of the stereoisomers of phenylalanine was established by du Vigneaud and Meyer (16) it became clear that the configurations assigned by Knoop were incorrect and that, as was subsequently shown by du Vigneaud and Irish (17), the acetylphenylalanine which was metabolically inert belonged to the d-series. The same authors demonstrated that the acetyl compound excreted after feeding of racemic phenylaminobutyric acid was derived from the l-acid and that it was the amino acid of unnatural configuration which underwent optical inversion. These findings re-established the experimental basis for the acetylation hypothesis. Knoop had originally suggested that acetylation was part of a synthetic process leading to acetyl amino acids by condensation of the corresponding keto acids with pyruvic acid and ammonia, in analogy to the in vitro reactions described by Erlenmeyer (18) and deJong (19). That acetylation was not necessarily linked to the amination of keto acids was shown by du Vigneaud et al. (20), who found that phenylamino butyric acid labelled with  $N^{15}$  retained during its conversion to the N-acetyl derivative nearly all of the  $N^{15}$  originally present, indicating that acetylation of the amino or imino acid had occurred. On the other hand,  $N^{15}$  was entirely replaced by normal nitrogen when similarly labeled d-phenylaminobutyric was converted to the acetyl l-acid, a result which pointed to the intermediate formation of phenylketobutyric acid. No need exists to postulate two separate

mechanisms for the acetylation of the isomeric amino acids if it is assumed that acetylation is preceded by inversion of the d- to the l-acid. The same authors also studied the acetylation of phenylaminobutyric acid in animals whose body fluids were enriched with D<sub>2</sub>O. One atom of deuterium was introduced at the  $\alpha$  carbon atom during the acetylation of both isomeric phenylaminobutyric acids. This can be explained readily in the case of the d-acid as resulting from the reductive amination of the intermediate keto acid by hydrogen derived from the labeled body fluids. On the other hand, introduction of deuterium at the  $\alpha$  carbon atom of the acetyl l-acid without simultaneous detachment of nitrogen, as shown by the retention of N<sup>15</sup>, can be visualized only if there exists an equilibrium between amino and imino acid which permits reversible hydrogenation and dehydrogenation. du Vigneaud et al. (20) therefore suggested a mechanism of acetylation in which condensation of the imino acid with pyruvic acid was the initial step, a scheme akin to that of Knoop (8) with the difference that the imino acid takes the place of keto acid plus ammonia. The formulation of the reaction was based on the assumption that pyruvic acid was the most likely source of acetyl. The subsequent demonstration that acetic acid is an acetylating agent for amines (1, 21) eliminated the necessity to associate the reversible hydrogenation-dehydrogenation, as indicated by the uptake of isotopic hydrogen at the  $\alpha$  carbon of acetyl amino acids, with the acetylation reaction. It is more likely that amino-imino acid equilibria are established independently of the acetylation reaction as a general property of amino acids. In some instances the acetylation of foreign amino acids of unnatural configuration seems to proceed more rapidly than oxidative deamination and subsequent inversion. S-benzyl d-cysteine (22), p-bromophenyl- and p-bromobenzyl d-cysteine (23) are in part converted to acetyl d-amino acids. These results provide additional evidence that acetylation can be independent of the amination of keto acids.

*Precursors of acetyl groups.* From the many attempts to identify the nature of the acetyl precursor by balance experiments with intact animals no clear picture has emerged. For instance, there are reports both that the dietary addition of acetate depresses (24, 25) and increases (26) acetylation of sulfanilamide. In balance experiments with intact animals a significant answer is to be expected only if the exogenous supply of precursor is the limiting factor. The probable acetyl precursors such as acetate, pyruvate, or acetoacetate must be available from metabolic reactions in much greater quantities than are required for the acetylation of the relatively small amounts of foreign amine which can be given to animals so that the dietary addition of precursors is unlikely to influence the yield of acetylated product. Direct evidence concerning the sources of acetyl groups for foreign amines has been obtained from feeding experiments with isotopically labeled test substances. Bernhard has administered to humans and rabbits deuterio acetic acid (21) and deuterio ethanol (27), as a source of acetyl for either sulfanilamide, p-amino benzoic acid or cyclohexylalanine (28). In all cases high concentrations of deuterium were found in the excreted acetyl derivatives, demonstrating the conversion of both acetate and ethanol to acetyl groups for aromatic amines and foreign  $\alpha$  amino acids. Similar

results were obtained with the stereoisomers of phenylaminobutyric acid by Bloch and Rittenberg (29, 30), who studied the quantitative aspects of the acetylation reaction. When equal amounts of labeled acetate are fed together with either d- or l-phenylaminobutyric acid, or with sulfanilamide or p-aminobenzoic acid, the excreted acetyl groups contain in all cases about the same concentrations of isotope. This indicates that acetic acid is an equally efficient source of acetyl groups for the two types of foreign amines represented by phenylaminobutyric acid ( $\alpha$  amino acid) and sulfanilamide (aromatic amine). From the data obtained it was concluded that acetic acid is the main source of acyl groups in the acetylation of foreign amines. The level of isotope in the excreted acetyl groups amounts to a relatively small fraction only of the labeled acetic acid administered. This dilution of isotope is believed to result from the mixing of the exogenous labeled acetate with acetic acid arising in intermediary metabolism (30).

The ability of acetic acid to acetylate amines is also clearly indicated by experiments with surviving tissues and tissue extracts. Acetylation of sulfanilamide has been investigated in slices of guinea-pig liver by Klein and Harris (1) and in homogenate and extracts of pigeon liver by Lipmann (2). In these investigations the aromatic amines, sulfanilamide and p-aminobenzoic acid, were used as acetyl acceptors because conjugation could be followed conveniently by determination of the disappearance of diazotizable amine. That acetyl sulfanilamide was the product formed was established by isolation. In these systems acetate was the most potent acetyl donor, while acetoacetate and pyruvate showed a lesser effect. The results suggest that the immediate reactant is either acetic acid itself or a C<sub>2</sub> compound which under these conditions arises more readily from acetate than from pyruvate or acetoacetate. Five times as much acetylsulfanilamide was formed under aerobic than under anaerobic conditions (2). The linking of acetylation with an oxidative process is further illustrated by the finding that the acetate effect is abolished by oxidation poisons such as cyanide or iodoacetate (1). Although oxygen does not enter into the formal equation of the acetylation by acetate, aerobiosis is required to supply the energy for the condensation. Lipmann has demonstrated that acetylation of sulfanilamide takes place also anaerobically if "energy rich" phosphate in the form of adenosine triphosphate is provided (2). It is not likely that the function of adenosine triphosphate consists in the formation of acetylphosphate since the addition of this compound does not elevate the level of anaerobic sulfanilamide acetylation. The enzyme system concerned in the acetylation of these aromatic amines is most abundant in pigeon liver and has been found to be extractable (2).

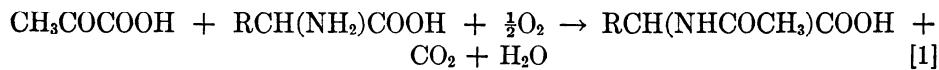
A different enzyme seems to be responsible for the acetylation of  $\alpha$  amino acids by acetate. Bloch and Borek (31) found, with the aid of deuterio acetate, acetylation not only of l-phenylaminobutyric acid but also of the natural amino acids, leucine and phenylalanine. The yields of acetyl amino acids were much higher with slices from rat liver than from those of guinea pig or pigeon. After incubation with labeled acetate, amino acid and non-isotopic acetyl amino acid as a carrier, the quantities of acetyl amino acid formed could be calculated from

the isotope content of the isolated acetyl amino acid. The amounts of acetyl compound obtained with phenylaminobutyric acid were much greater than with the two natural amino acids, presumably because the acetyl derivative of the foreign amino acid is not further metabolized while the acetylated natural amino acids seem to be short-lived intermediates. Thus, under the same experimental conditions the acetyl derivative of a natural amino acid, acetyl glycine, was found to be deacetylated.

These results indicate that aromatic amines and both natural and foreign  $\alpha$ -amino acids effectively utilize acetic acid as a source of acetyl groups. It is evident that whatever the nature of the C<sub>2</sub> compound which is the immediate acetyl precursor, it can readily be formed from acetic acid itself. The effect of adenosine triphosphate on the acetylation reaction observed by Lipmann (2) suggests that the condensation between the carboxyl group of acetic acid and the amine involves the elimination of phosphoric acid rather than of water. Since the effect of adenosine triphosphate cannot be attributed to the intermediary formation of acetylphosphate, the possibility remains to be explored that phosphorylated sulfanilamide or an acetyl derivative of adenosine triphosphate may be formed as intermediates.

The existence of a second mechanism of acetylation and of another source of acetyl groups is indicated by results obtained with labeled alanine (29, 30). Alanine which contains deuterium at the  $\alpha$  and  $\beta$  carbon atoms causes the excretion of deuterio acetyl groups when phenylamino butyric acid is simultaneously administered, but only a slight excess of isotope is found in the acetylation product when sulfanilamide or p-aminobenzoic acid are fed as acetyl acceptors. Since alanine, in contrast to acetate, provides acetyl groups for  $\alpha$  amino acids only, its effect cannot be explained on the basis of intermediate formation of acetic acid. The effect of alanine may reasonably be ascribed to the condensation of a C<sub>3</sub> compound, presumably pyruvic acid, with an imino acid and subsequent decarboxylation of the condensation product to an N-acetyl derivative. In this case the failure of alanine to acetylate aromatic amines can be readily understood since imino acids cannot arise from aromatic amines.

The above result which provides experimental evidence for the acetylation schemes suggested by Knoop (8) and du Vigneaud et al. (20) is of interest in several respects. The reaction:

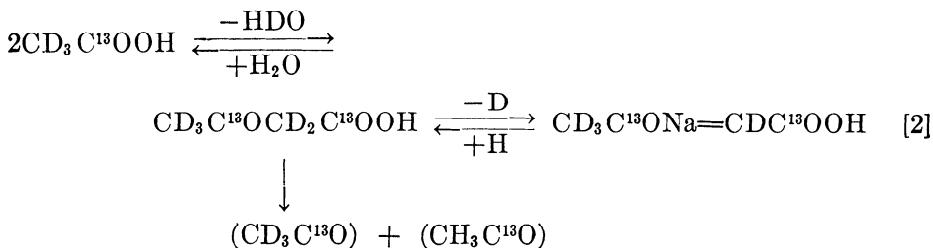


can be assumed to proceed with an overall decrease of free energy so that it becomes unnecessary to postulate the intermediate formation of an active C<sub>2</sub> compound as the immediate precursor of N-acetyl groups. This is in contrast to the acetylation of amines by acetic acid which is an endergonic process and therefore requires coupling with an energy yielding reaction. The conversion of pyruvic acid to acetyl amino acid, by effecting the degradation of a three carbon compound to a C<sub>2</sub> unit without intermediate formation of free acetic acid, offers a possible mechanism for the conversion of pyruvate to the oxidation level of

acetate, a step in the oxidation of carbohydrate which is little understood at present.

That there may exist a connection between acetylation reaction [1] and the formation of acetoacetate from pyruvate has been pointed out by Krebs (32). The fact that ketone body formation from pyruvate in liver is greatly enhanced in the presence of ammonia (33, 34) can be explained on the basis of acetylaminoo acid formation from keto acid, ammonia and pyruvate, and subsequent condensation of acetyl groups to acetoacetate. Formation of an N-acetyl derivative from pyruvate has so far been demonstrated only with phenylamino-butyric acid but not with natural amino acids. Also, the ability of N-acetyl groups to yield acetoacetate remains to be tested.

The direct participation of acetoacetate as an intermediate in the acetylation of amines by acetic acid has been considered but appears unlikely in view of the following findings (29, 30): deuterioacetyl groups are formed from butyric acid labeled with heavy hydrogen either at the  $\alpha$  or the  $\gamma$  carbon atoms, indicating that both two carbon moieties of the intermediate acetoacetic acid are employed for acetylation. Secondly, deuterioacetic acid which also contains C<sup>13</sup> at the carboxyl position affords N-acetyl groups in which the relative proportion of C<sup>13</sup> and D remains unchanged. The formation of acetoacetate from such doubly labeled acetate would entail replacement of deuterium by normal hydrogen, but no loss of C<sup>13</sup>, as the result of the following reactions:

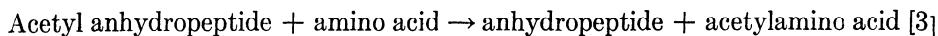


Any acetyl formed by way of acetoacetate should therefore have a diminished D:C<sup>13</sup> ratio. Since the conversion of acetate to N-acetyl is not accompanied by any loss of carbon-bound deuterium, acetoacetate may be ruled out as an intermediate step. The fact that the D:C<sup>13</sup> ratio is not affected in a reaction in which acetic acid is utilized also speaks against a rapidly occurring interconversion of acetate and acetoacetate in the intact animal. The significance of this finding with regard to fatty acid catabolism will be discussed later.

*Metabolism of acetylaminoo acids.* The occurrence of a process by which acetyl groups can be distributed between amino acids is suggested from data obtained with labeled acetylaminoo acids (35). These experiments were carried out in order to determine whether the acetyl derivatives of natural amino acids can be biologically deacetylated. Any acetic acid liberated from these compounds should become demonstrable by its capacity to acetylate foreign amines. When the deuterio acetyl derivatives of glycine, alanine, leucine or glutamic acid were fed together with phenylaminobutyric acid, deuterium was found in the excreted

acetyl phenylaminobutyric acid, but the level of isotope was very much higher than that obtained after feeding of equivalent quantities of labeled acetic acid. This result was interpreted as showing that the foreign amino acid had received acetyl by direct transfer from the administered acetylamino acid and that in the process no intermediate formation of free acetic acid had occurred. The high isotope concentrations in excreted acetylamine were not obtained when p-amino benzoic acid was the foreign amine. In the transfer of acetyl groups an  $\alpha$  amino acid is therefore necessary as an acetyl acceptor. Only the acetyl derivatives of natural amino acids can serve as acetyl donors; the labeled acetyl derivatives of d-alanine, d-leucine and sarcosine failed to yield deuterio acetyl groups. These results paralleled those obtained with acetyl d-amino acids in growth experiments and substantiate the conclusion that no biological mechanism is available for the deacylation of acetyl d-amino acids. The fact that  $\alpha$ -amino acids are necessary both as acetyl acceptors and as acetyl donors may perhaps indicate that the transfer reaction is coupled with dehydrogenation at the  $\alpha$  carbon atom of the amino acids and that in order to allow the reaction to occur the steric configuration of the hydrogen at the  $\alpha$  carbon must be that of the natural amino acids. Data on the acetyl transfer reaction have so far been obtained only with phenylaminobutyric acid as acetyl acceptor. *In vivo* the acetyl derivatives of natural amino acids do not accumulate sufficiently to permit their isolation. Transacetylation as a process which operates between two natural amino acids therefore remains to be demonstrated.

Organic chemical reactions representing intermolecular transfer of acetyl groups to amino acids have been known from the investigations of Bergmann et al. Bergmann, du Vigneaud and Zervas (36) found that the acetyl groups of diacetyl diketopiperazine are readily shifted to free amino acids. The reaction proceeds in aqueous solution at room temperature:

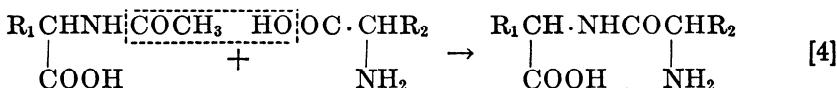


Acetyl transfer was also shown to occur with a histidine derivative which contained the acetyl substituent at one of the imidazole nitrogen atoms (37). Evidently an acetyl group is more reactive when linked to a secondary nitrogen and its transfer to a primary amino group *in vitro* will therefore occur spontaneously. This raises the possibility that the acetyl donor concerned in the biological transfer of acetyl groups may be an N-acetylpeptide rather than a free acetylamino acid. On the basis of these model reactions Bergmann and Zervas (37) suggested that acetylamino acids might play a rôle in the biological formation of peptide bonds. This hypothesis assumes added significance in view of the demonstration that the acetyl derivatives of natural amino acids are biologically formed (31) and that a mechanism exists for the shifting of acetyl groups from one amino acid to another (35).

The formation of an acetylamino acid presumably requires a very similar quantity of energy as the formation of a peptide bond, i.e., 2.5 to 3.0 K cal. (38). This energy can readily be supplied by the acetylation mechanism [1] which involves the conversion of pyruvic acid to N-acetyl. On the other hand, it is

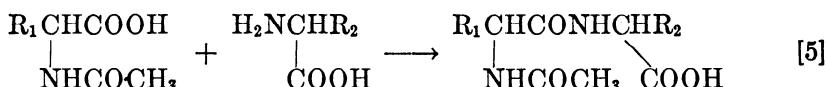
not clear which energy yielding step facilitates the acetylation of amino acids by acetate.

Acetyl amino acids may react with free amino acids in two ways: 1, by acetolysis:



This reaction should involve only a small change of free energy.

Or 2,



The second reaction, which would yield an acetylpeptide, is analogous to the acetylation of amino acids by acetic acid, an acetamino acid being the acetylating agent instead of acetic acid. It may be pointed out that as a result of acetylation, the amphoteric amino acids are converted into strong acids (39).

The ability of the animal organism to utilize acetyl amino acids is also illustrated by the finding that, with the exception of  $\alpha$ -N-acetyl lysine (40), the acetyl derivatives of essential amino acids are nutritionally equivalent to the free amino acids. This has been tested in growth experiments with the acetyl derivatives of tryptophane (41, 42), phenylalanine (43), and histidine (44). The ability of acetyl glycine to raise the level of hippuric acid excretion in rabbits above the normal suggests conversion of the acetyl derivative to the free amino acid (45). In those instances in which the acetyl amino acid can take the place of an indispensable amino acid, the corresponding  $\alpha$  keto or  $\alpha$  hydroxy acids will likewise be capable of supporting growth (46). It is therefore not clear whether regeneration of the free amino acid from the acetyl derivative involves direct deacetylation by hydrolysis or whether it is due to a process which regenerates the amino acid by way of the  $\alpha$ -keto or  $\alpha$ -hydroxy acid. The observation that  $\alpha$ -N-acetyllysine cannot support the growth of rats on a lysine deficient diet has been interpreted by Neuberger and Sanger (40) as suggesting rapid oxidation of the  $\epsilon$  amino group of lysine prior to deacetylation. However, it is also possible that the  $\alpha$ -keto acid is formed directly without passing through free lysine as an intermediate. The  $\alpha$ -keto acid of lysine is not known, but it may be presumed to be incapable of substituting for lysine in supporting growth because the  $\alpha$ -nitrogen of lysine, in contrast to that of other amino acids, cannot be replaced metabolically by nitrogen from other sources (47) and because d-lysine cannot support growth (48). On the other hand, Neuberger and Sanger (40) found  $\epsilon$ -N-acetyllysine to be nutritionally equivalent to lysine, suggesting a process of metabolism in which the acetyl group is removed by hydrolysis to regenerate free lysine. In agreement with this observation Bloch and Rittenberg found deuterio  $\epsilon$ -N-acetyllysine to yield acetyl groups for the acetylation of foreign amines with an isotope content to be expected from hydrolysis of the acetyl amino acid (unpublished experiments).

The enzyme systems catalyzing deacetylations show specificity with respect to the spatial configuration of the acetyl amino acids. Thus the acetyl derivatives of d-tryptophane (42, 49), d-phenyl alanine (17) and d-leucine (35) are metabolically inert; acetyl d-phenylalanine has been recovered unchanged from the urine of the experimental animals (17). The sulfur of acetyl d-cystine is resistant to oxidation while acetyl l-cystine is readily metabolized (50). The unavailability of acetyl d-amino acids must be due to the inability of animal tissues to deacetylate because the corresponding free d-amino acids can be utilized and can replace nutritionally their optical antipodes (49, 51, 52). This is further illustrated by the failure of the deuterio acetyl derivatives of d-alanine and d-leucine to yield labeled acetyl for the acetylation of foreign amines (35). Some significance may be attached to the fact that whereas deamination occurs readily with both stereoisomers of most amino acids, only the acetyl amino acids of natural configuration are metabolized by animal tissues.

Acetylation may be associated with the formation of amino acids from keto acids and ammonia but it can also occur subsequent to amino acid synthesis. It is thus conceivable that acetylation may be concerned in the catabolism of amino acids. The following findings are of interest in this connection. The observation already mentioned of Neuberger and Sanger (40) that  $\alpha$ -N-acetyl-lysine cannot replace lysine for support of growth indicates that the compound is not deacetylated but that it may be directly converted to the nutritionally ineffective  $\alpha$  keto acid. Secondly, it has been found that on feeding of acetyl-l-phenylalanine to rats, phenylacetic acid can be isolated from the urine in the form of phenaceturic acid. (Unpublished observation of the author.) Since this compound was not obtainable after the feeding of free l-phenylalanine it would appear that the acetyl derivative was deaminated more readily than the free amino acid.

**FORMATION OF ACETIC ACID.** The first suggestions of a formation of acetic acid in quantity in animal metabolism were contained in experiments of Knoop (53) and Dakin (54) on the biological degradation of phenyl-substituted fatty acids. The excretion of either phenylacetic acid or benzoic acids (or their conjugation products with glycine), depending on the number of carbon atoms in the aliphatic chain of the test substance, was most reasonably explained as resulting from the successive removals of two carbon fragments, by hydrolysis of an intermediate  $\beta$  keto acid. The exact nature of the two carbon fragments could not be ascertained, but chemical considerations pointed to acetic acid or an acetyl compound. Acetic acid has been proposed repeatedly as an intermediate in the main path of carbohydrate oxidation. Thunberg (55) suggested that oxidative decarboxylation of pyruvic acid gave rise to acetic acid, two molecules of which were dehydrogenated to form succinic acid. In the course of their metabolism, several amino acids form intermediates which are identical with products of either carbohydrate or fatty acid breakdown, pointing to protein as a potential source of acetic acid. Thus it was conceivable that the metabolic paths of the three major dietary and tissue components, fat, carbohydrate and protein, would converge at the two carbon stage and that, once this

stage had been reached, only one common mechanism of oxidation would be required. It is interesting to note that the principal aspects of this theory of intermediary metabolism which, at the time, rested on little experimental evidence, have required revision in a few details only. Experimental evidence in its favor continues to accumulate.

*The occurrence of acetic acid in animal tissues.* As an intermediate in the main chain of metabolic reactions, acetic acid should continually arise in large quantities. Analysis of tissues and body fluids reveals that in the equilibrium state, acetic acid is present in quantities which are barely detectable (56). Acetic acid in the form of its dinitrophenylhydrazone has been isolated from large quantities of beef liver (57), but it is not clear whether the material thus obtained was an artefact formed during the isolation procedure. Specific methods for the determination of small quantities of acetic acid in biological mixtures are not available; it may also escape detection because it seems to be firmly bound to protein in biological media (58, 59). Nevertheless, even if acetic acid were found to be practically absent, the suggested rôle of acetic acid as a major product of catabolism would not be contradicted. The condition which has to be satisfied, and which applies to all metabolites which lie on the main metabolic paths, is a capacity of the tissue to metabolize the intermediate as rapidly as it is formed. The concentration of the metabolite at any one time will then be irrelevant. The insignificant concentration of acetic acid and other fatty acids of intermediate size in animal tissues has been cited as evidence against the theory of  $\beta$  oxidation (60). If this objection were valid, the same reasoning would apply to numerous commonly accepted intermediates of protein and carbohydrate metabolism, because the demonstration of their presence in normal tissues has not been experimentally feasible.

Evidence for the occurrence of a metabolite which does not normally accumulate in sufficient amounts to permit analytical detection can be obtained with the aid of the isotope dilution method. If the isotopic analogue of a suspected intermediate is administered to an animal, or added to an isolated system, and subsequently recovered either as such or in combined form, a diminished isotope content of the isolated material will indicate that the test substance had merged with identical unlabeled molecules formed in the tissues. Bernhard, in an investigation of the acetylation of foreign amines with labeled test substances (21) found that the acetyl groups of excreted acetyl sulfanilamide contained 5 per cent to 20 per cent as much isotope as the deuterio acetic acid added to the diet. He concluded that the fraction of acetyl groups contributed by exogenous acetate was related to the difference of isotope content in the test substance and excreted acetyl. These findings suggested that the acetyl precursor consisted of a mixture of dietary and endogenous acetic acid. The possibility that the acetic acid excreted by the animal in the form of acetylamine was a representative sample of the acetate existing in the metabolic pool offered an experimental approach to a quantitative determination of this metabolite in the intact animal. From such experiments, Bloch and Rittenberg (30) have concluded that the isotope dilution obtaining in the acetylation process can be attributed to the merging of labeled

dietary acetate with acetate arising in metabolism. The magnitude of the dilution factor indicates that the quantities of acetic acid produced are large.

In order to ascertain the correctness of this conclusion it was necessary to consider the following sources of isotope dilution apart from that caused by endogenous acetate: 1, rate of the acetylation reaction; 2, labilization of the C—H bond in the methyl group of deuterio acetic acid; 3, acetylation by precursors other than acetic acid.

1. Since the acetyl derivatives of foreign amines are not stored by the animal but excreted into the urine soon after their formation, the rate of the reaction need not be considered. The isotope concentration of excreted acetyl is the same irrespective of the length of time during which labeled acetate is administered. 2. In one of the acetylation experiments, a preparation of acetic acid which contained deuterium in the methyl group and a carboxyl group labeled by C<sup>13</sup> was administered simultaneously with phenylaminobutyric acid. The acetyl group of the excreted acylamino acid contained the two isotopic markers in much smaller concentrations, but the ratio of C<sup>13</sup>:D had remained unchanged. This finding eliminates loss of deuterium as a source of isotope dilution and also attests to the biological stability of the C—H bonds in the methyl group of acetic acid. 3. If the acetyl groups were derived not only from acetic acid but also from other precursors, such acetyl groups would be non-isotopic and hence dilute the deuterio acetyl groups which originate from labeled acetate. Evidence for the contention that acetate may be the only source of acetyl groups for aromatic amines (sulfanilamide, p-aminobenzoic acid) and the major one in the acetylation of  $\alpha$  amino acids has been discussed above. It is based on the finding that in identical feeding experiments, acetic acid effects much higher isotope concentrations in excreted acetyl than any other compound tested. According to Bernhard (27), labeled ethanol is a better source of acetyl than acetic acid, but in the experiments of Bloch and Rittenberg (29) ethanol and acetate were equally effective. On the basis of available evidence it seems likely that alcohol is rapidly oxidized to acetic acid and thus becomes a source of acetyl groups. Still, the possibility exists that a C<sub>2</sub> compound which is readily available from both alcohol and acetate is the immediate acetylating agent.

The finding that alanine is a source of acetyl groups for foreign  $\alpha$  amino acids but not for foreign aromatic amines has been attributed to a condensation of pyruvic acid with the imino acid derived from phenylaminobutyric acid. While these results establish the existence of a second source of acetyl groups for  $\alpha$  amino acids, they do not invalidate the contention that in the acetylation of aromatic amines acetic acid serves as the sole source of acetyl groups. It was therefore reasonably well established that in feeding experiments with labeled acetate the change in isotope concentration occurring in the acetylation reaction provides a direct measure of endogenous acetate production. By applying the equation for isotope dilution (61), the quantity of acetate formed by the rat in a 24-hour period was calculated to be 15 to 20 mM per 100 grams, or approximately one per cent of the body weight. This value could be in error in either direction if the ingested acetic acid failed to mix adequately with the acetate

of the metabolic pool, in which case either the dietary material or the endogenous acetate might be employed preferentially for acetylation. However, the following considerations lend support to the correctness of the above calculation: the isotope concentration of excreted acetyl was found to be directly proportional to the total quantity of labeled acetate administered per unit of body weight, and hence the calculated value for acetate production *in vivo* is independent of dosage variation over a wide range. This finding points to a rapid merging of dietary acetate and the acetic acid formed in the tissues.

*In vitro* studies by Klein and Harris (1) indicate that the acetylation reaction is confined to the liver. The sample of acetate which becomes trapped by forming acetylamine is therefore representative only of the hepatic acetate pool. Of the acetic acid of extrahepatic origin, only the fraction which escapes oxidation at the site of formation and is dispatched to the liver will be measured in the acetylation reaction. The resultant error cannot be assessed at the moment, because it is not known to what extent acetyl-yielding reactions take place in extrahepatic tissues.

Lorber et al. (62) perfused the isolated heart with labeled acetic acid and found that the latter was apparently diluted by normal acetate in amounts corresponding to those calculated for the intact rat (30).

R. Q. measurements of animals in the fasting state (63) and after hepatectomy (64) have been interpreted as indicating fatty acid oxidation in organs other than the liver. Stadie (65) has calculated that in the animal depleted of carbohydrate stores, the energy requirements cannot be met by hepatic production of ketone bodies. The occurrence of extrahepatic fat oxidation is illustrated by experiments of Lehninger, who showed that palmitic and octanoic acids are metabolized by a heart muscle preparation to yield succinate and ketoglutarate (66). An independent estimate of the size of the metabolic acetate pool has been made on the assumption that a molecule of fatty acid of average size ( $C_{16}$ ) yields on degradation eight molecules of acetate. With the aid of available data on rates of fatty acid regeneration (67) it is possible to calculate how much acetate should arise in a given period of time if fatty acids were the only source. The quantity thus calculated agrees closely with that obtained experimentally by isotope dilution measurements.

*Acetic acid formation from fatty acids.* Fatty acid oxidation<sup>1</sup> has been investigated in a variety of systems, viz., in the fasting or diabetic animal, in perfused livers, in various liver preparations, and in the intact animal with the aid of labeled test substances. Under all conditions except those mentioned last, acetoacetic acid is the principal product of the oxidation process. Leloir and Muñoz (68) detected acetic acid in the course of octanoate oxidation by liver, but the amount was insignificant compared with the quantities of ketone bodies which accumulated. Deuel and co-workers (69, 70) found a uniform increase of ketone body excretion with increasing chain length of the fatty acid, administered as salt or ethyl ester, to starving rats. In isolated liver, the yields of

<sup>1</sup> For a detailed consideration of this subject the reader is referred to the review by Stadie (60).

ketone bodies per molecule of fatty acid obtained by different investigators differ widely (71 to 74), depending apparently on the previous treatment of the liver tissue. *In vitro*, fatty acids appear to become increasingly resistant to oxidation as the length of the chain increases. The depressing effect of higher fatty acids on the respiration of isolated tissue, noted by Quastel and Wheatley (71), has been attributed to their surface activity. Lehninger has been able to show, however, that oxygen uptake can be restored and that the higher fatty acids can be oxidized by addition of adenosine triphosphate to the liver system (74). Under the experimental conditions of this investigator the carbon atoms of some even and odd numbered fatty acids ( $C_8$ ,  $C_7$ ) could be accounted for as acetoacetate in nearly quantitative yields.

Since acetoacetic acid accumulates as the principal product of fatty acid oxidation, it is not surprising that acetoacetate rather than acetate has frequently been emphasized as the primary oxidation product. On the other hand, recent experiments have furnished clear evidence for the correctness of the principle of  $\beta$  oxidation as proposed by Knoop (53). Stepwise degradation of a biologically occurring fatty acid by elimination of two carbon atoms was first demonstrated by Schoenheimer and Rittenberg (75). Palmitic acid isolated from the tissue fat of animals which had received deuterio stearic acid had a sufficiently high isotope content to show that it arose directly from the  $C_{18}$  acid by shortening of the chain. Their data indicated that myristic acid had been similarly formed by subsequent elimination of two carbon atoms from palmitic acid. The further degradation could not be studied because tissue fat contains only traces of the acids with less than 14 carbon atoms.

The two carbon fragment which is detached from the fatty acid chain has not been identified as such, although its intermediate formation has been clearly established. Weinhouse, Medes and Floyd (76) have investigated the oxidation of octanoic acid which was labeled at the carboxyl group with  $C^{14}$ , in liver slices. The acetoacetic acid which accumulated as the main product, contained labeled carbon in nearly equal concentrations at the carbonyl ( $\beta$  carbon) and carboxyl positions. Acetoacetate with such isotope distribution could not have resulted from the splitting of the  $C_8$  acid into two four carbon compounds (multiple alternate oxidation), nor could it have been formed exclusively from carbon atoms 5 to 8 of the octanoic acid (classical  $\beta$  oxidation). Acetoacetate must have been formed, at least in part, by random condensation of  $C_2$  fragments.

The ability of fatty acids to yield acetyl groups in the intact animal can be tested by exploiting the fact that foreign amines are converted *in vivo* into metabolically inert acetyl derivatives. Since acetic acid is an effective source of such acetyl groups, the assumption can be made that the appearance of labeled acetyl amine after feeding of labeled precursor is a measure of acetyl formation. The following deuterio-fatty acids were found by Bloch and Rittenberg (29) to be sources of deuterioacetyl groups: butyric acid, n-valeric acid, isovaleric acid and myristic acid; propionic and undecylic acids failed to yield acetyl. The results obtained were found to conform with the assumption that  $\beta$  oxidation and removal of acetyl groups from the  $\beta$  keto acids was the principal event in the

metabolism of the fatty acids. It is clear that deuterium is not always an adequate label for the study of carbon chain interconversions, and the results obtained on acetyl formation are in most cases qualitative only. All deuterium attached to the  $\beta$  carbon atoms will be lost by oxidation and further loss of deuterium is to be expected due to keto-enol tautomerism involving the methylene hydrogens adjacent to the keto groups. Thus,  $\alpha, \beta$  dideuteriobutyric acid yields acetyl of much lower isotope concentration than butyrate which contains deuterium at the  $\beta$  and  $\gamma$  positions. If acetoacetate is the intermediate in the butyrate-acetyl conversion, loss of deuterium from the labile  $\alpha$  position and therefore a lowered isotope content of the acetyl fragment derived from the carboxyl and  $\alpha$  carbon atom of butyric acid would result. The behavior of the two preparations of deuterio butyric acid suggests breakdown of the intermediary  $\beta$  keto acid into two identical acetyl fragments.

The various  $\beta$  keto acids which are formed successively from the higher fatty acids will suffer the same loss of deuterium at the  $\alpha$  carbon as the  $\alpha$  fragment of butyric acid, so that only the deuterium bound to the terminal carbon remains in stable linkage. In the intact animal, the long chain fatty acids, in contrast to those having a small number of carbon atoms, are only partly catabolized immediately to yield acetyl groups. For example, a large portion of administered myristic acid will be deposited in the tissues as such and will also be converted to palmitic and stearic acids by chain elongation. In this case the deuterium content of excreted acetyl will not be a quantitative measure of the amounts of acetyl produced.

*Metabolism of odd numbered fatty acids.* The odd numbered straight chain fatty acids are not encountered in animal metabolism but they are oxidized in vivo and in vitro as readily as the corresponding even numbered fatty acids. In isolated liver (72) and in the intact animal (77) the fatty acids possessing an odd number of carbon atoms (except propionic acid) are precursors of ketone bodies although the quantities produced are smaller than those afforded by the neighboring fatty acids with an even number of carbon atoms. Propionic acid is presumably oxidized to a three carbon compound which can be converted quantitatively to carbohydrate. Bloch and Rittenberg have concluded that pyruvic acid cannot be an intermediate in this conversion since deuteriopropionic acid, in contrast to labeled alanine, failed to provide acetyl groups in the acetylation of phenylaminobutyric acid (29). Deuterio n-valeric acid formed labeled acetyl to an extent which indicated that one two-carbon fragment arose per mole of valerate. Cohen found that isovaleric acid is a much more potent source of acetoacetate in liver slices than n-valerate (73). In the intact animal labeled isovalerate yielded more deuterioacetyl than equivalent amounts of the n-valerate (78). From the data obtained, it could not be decided whether the breakdown of this branched chain acid proceeds by way of demethylation to butyric acid or by initial oxidation at the tertiary carbon atom to acetone and a C<sub>2</sub> fragment. Undecylic acid labeled with deuterium at the 10 and 11 positions did not yield deuterio acetyl.  $\beta$  oxidation of this eleven carbon acid may be visualized to lead eventually to a three carbon fragment which contains the

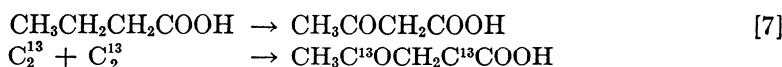
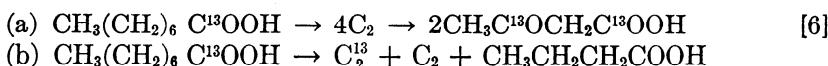
isotopic hydrogen but which, like propionic acid, would not be degraded directly to a C<sub>2</sub> unit. The behavior of the odd numbered fatty acids can be readily understood if it is assumed that their metabolism proceeds in a fashion analogous to that of the even-numbered fatty acids with the difference that their terminal three carbon atoms are, like propionic acid, converted to carbohydrate. From an odd numbered acid having n carbon atoms, only n - 3 carbon atoms will then be available for ketone body formation so that less acetoacetate will be formed per molecule than from the neighboring even numbered fatty acid.

The demonstration of the ketogenic effect of n-valeric acid (72, 77) has had an important bearing on the development of more recent theories of fat oxidation. This finding could not be adequately explained by either classical  $\beta$  oxidation or by the hypothesis of multiple alternate oxidation. MacKay et al. (77) suggested that the five carbon chain of valerate was degraded to a three carbon and a two carbon unit and that two of the latter combined to yield acetoacetate. It became evident therefore that ketone bodies could be formed by recondensation of two carbon fragments split off from the fatty acid chain as well as by direct oxidation.

*Formation of acetyl from amino acids.* It is generally believed that  $\alpha$  amino acids are catabolized by oxidative deamination to  $\alpha$  keto acids and subsequent oxidative decarboxylation to acids containing one carbon atom less. In this case amino acids which yield saturated fatty acids as intermediates should be sources of acetyl groups. This possibility has been studied with the amino acids leucine and valine which contained carbon chains labeled by deuterium (78). Deuterioleucine yielded labeled acetyl groups to the same extent as isovaleric acid indicating that this acid was formed as an intermediate. The ketogenic effect of leucine (73, 79) may be explained on the same basis. Valine and isobutyric acid both are nonketogenic and are also ineffective as sources of acetyl groups. No information is available concerning the steps by which isobutyric acid is further metabolized except that three of its four carbon atoms are convertible to glycogen (80). It should be of interest to ascertain whether the ketogenic action of phenylalanine and tyrosine is associated with acetyl formation, particularly whether the ring structure of these amino acids can be degraded to two carbon units.

*Mechanisms of fatty acid oxidation.* A consideration of all pertinent data obtained in experiments with intact animals and with *in vitro* systems leads to the conclusion that successive  $\beta$  oxidation, followed by removal of C<sub>2</sub> fragments, is the principal event in fatty acid oxidation. For a number of years undue emphasis was placed on experimental observations which appeared to contradict  $\beta$  oxidation as originally proposed by Knoop. It should be pointed out that the data which led to a criticism of the theory of  $\beta$  oxidation were obtained with either isolated tissues or starving or diabetic animals. Since under these conditions acetoacetic acid and not acetic acid accumulates, the ketone bodies came to be regarded as primary oxidation products not only of the terminal four carbon atoms but of the entire fatty acid chain. Moreover, the theory of multiple alternate oxidation was advanced as a mechanism for fat oxidation

because, *in vivo* and *in vitro*, the fatty acids containing six and more carbon atoms gave a higher yield of acetoacetic acid than butyric acid, while Knoop's theory provided for the formation of only one mole of keto acid regardless of the length of the chain. However, the formation of ketone bodies in excess of one mole per mole of fatty acid is not at variance with  $\beta$  oxidation if it is ascribed to the recondensation of acetic acid to acetoacetic acid. The occurrence of this reaction was early demonstrated by Loeb (3) and Friedemann (81), who observed acetoacetate formation on perfusing liver with acetate. This finding has been confirmed repeatedly and under a variety of conditions, viz., by Jowett and Quastel and by Leloir and Muñoz in liver slices (72, 68), by Monguio and by MacKay et al. in intact animals (4, 5), and with C<sup>13</sup> labeled acetate in starving animals by Swendseid et al. (82) and by Medes et al. in liver slices (83). It was recognized by MacKay et al. (5) that the synthetic formation of acetoacetate from acetate provided a basis for a theory of fat oxidation which retained Knoop's basic concept of  $\beta$  oxidation and ascribed the formation of "excess" acetoacetate to a subsequent event, namely, a recombination of two molecules of acetate. The data of Weinhouse et al. with carboxyl labeled octanoic acid (76) demonstrate that acetoacetate can be formed by random coupling of C<sub>2</sub> fragments which are split off from the fatty acid chain. There are, however, two possible pathways which conform with the observed isotope distribution:



Reaction (a) does not indicate the mechanism which yields the four C<sub>2</sub> fragments from the fatty acid chain; it implies that all of the acetoacetate is of synthetic origin. Reaction (b) is a modified version of classical  $\beta$  oxidation and retains the view that the four terminal carbon atoms are a direct source of acetoacetic acid. In this case the accumulated acetoacetic acid would be a mixture of "primary" and synthetic molecules. In similar experiments with carboxyl labeled butyric acid the resulting acetoacetate contained an unequal distribution of C<sup>13</sup> at the carboxyl and carbonyl carbon atoms (85). This acetoacetate could therefore not have been formed by random coupling of two equivalent C<sub>2</sub> fragments. Medes et al. (85) have pointed out that their data can be represented by the following equation:



In this case rapid interconversion of acetate and acetoacetate has to be postulated. This is true for the synthesis of acetoacetate in liver (84) and kidney (86). The reverse reaction was found by Lehninger (59) to proceed slowly in muscle mince, but it is not clear whether in liver and kidney acetoacetate is split at a sufficient rate to account for the redistribution of isotope in accordance with the above equation.

A similar explanation may apply to the results obtained by Morehouse (87) and by Morehouse and Deuel (88), who fed two preparations of deuterio butyric acid and deuterio caproic acid to starving rats and isolated  $\beta$ -hydroxybutyric acid from the urine. The excreted hydroxybutyrate contained nearly twice as much deuterium when  $\beta$ - $\gamma$ -dideuteriocaproate was fed as the acid excreted after administration of caproate labeled at the  $\alpha$  and  $\beta$  positions. It is evident that a splitting of the hexanoic acid into three two carbon units followed by recondensation to acetoacetic acid should yield hydroxybutyric acid containing the same isotope concentration in both cases. On the other hand, if the excreted  $\beta$ -hydroxybutyric acid was derived mainly from "primary" acetoacetate representing the intact chain of carbon atoms 3 to 6 of caproate, and was formed only in part by recondensation of  $C_2$  units, then  $\beta$ - $\gamma$ -dideuteriocaproate should yield a hydroxybutyric acid of higher deuterium concentration than  $\alpha$ - $\beta$ -dideuteriocaproate.

Stadie (60) has pointed out that the data obtained by Weinhouse et al. with labeled octanoate are not inconsistent with the theory of multiple alternate oxidation if the random distribution of labeled carbon is the result of a splitting and reformation of acetoacetate. Since it is agreed that the formation of a  $C_2$  fragment is an obligatory step in the breakdown of fatty acids, it remains to be decided whether acetoacetate is the primary oxidation product or whether its formation represents an alternative path for the disposal of  $C_2$  fragments which are initially formed.

Some indications as to the rôle of acetoacetate in fatty acid oxidation are contained in experiments on the acetylation of foreign amines by acetic acid (30). It has been pointed out that the results from these experiments eliminate acetoacetic acid as an intermediate in the conversion of acetate to N-acetyl groups. From the same analytical data it can be concluded<sup>2</sup> that in the intact animal a rapid interconversion of acetate and acetoacetate is unlikely to occur. Doubly labeled acetic acid ( $CD_3C^{13}OOH$ ), when fed and recovered as acetyl phenylaminobutyric acid, retains deuterium and  $C^{13}$  in identical relative concentrations. Had acetate been converted to acetoacetate and regenerated, replacement of deuterium by normal hydrogen and loss of deuterium by enolization should have altered the D: $C^{13}$  ratio. The accumulation of acetoacetic acid in fatty acid oxidation in vitro, or in the urine of animals in ketosis may therefore be ascribed to circumstances which interfere with the normal disposal of acetyl or acetate. The isolated liver or liver preparations as ordinarily employed contain little available carbohydrate, and therefore lack the requisite dicarboxylic acids for the oxidation of the intermediates of fatty acid breakdown. Liver tissue appears to have only a limited capacity to oxidize fat beyond the  $C_2$  stage but dispatches the products to extrahepatic tissues for complete combustion. In the isolated liver acetyl will therefore accumulate and become stabilized in the form of acetoacetate. This point is clearly illustrated by the experiments of Lehninger (89) who found that in preparations of washed liver cells, acetoacetate is the

<sup>2</sup> The author is indebted to Prof. D. L. Thomson for having called attention to these implications contained in the experimental data.

sole product of octanoate oxidation, but that on addition of fumarate the quantities of acetoacetate are diminished in favor of  $\alpha$ -ketoglutarate, succinate, and citrate. It becomes unnecessary then to assume that acetoacetate lies on the main path of fat oxidation. Acetoacetic acid is utilized under a variety of conditions, but this is probably preceded by its splitting into two carbon units.

*Formation of acetyl from pyruvate.* There exists at present no conclusive evidence to indicate whether pyruvic acid enters the citric acid cycle by combining directly with oxaloacetic acid or whether this condensation is preceded by a conversion of pyruvate to an intermediate which has the oxidation level of acetic acid. Formation of acetate from pyruvate can be demonstrated to occur in animal tissues, either aerobically (90, 91, 92) or anaerobically by dismutation (90, 91, 93) but this reaction is not believed to represent an important pathway, because under the same conditions acetate itself is oxidized relatively slowly. It is considered more likely that the oxidation product of pyruvate is represented by a two carbon compound which is metabolically more reactive than acetic acid itself. A phosphoroclastic splitting of pyruvate into acetyl-phosphate and formate (or  $\text{CO}_2 + \text{H}_2$ ) as it occurs in bacteria (94) has not been observed in animal tissues.

According to Barron et al. (95), acetate can be shown to accumulate when liver or kidney slices oxidize pyruvate in the presence of fluoroacetate. According to these investigators, this compound seems to inhibit the oxidative disappearance of acetic acid by competing with acetate for the enzyme. The accumulation of acetate indicates that fluoroacetate may exert its effect by blocking condensations involving the reactive acetyl compound, which may then become stabilized in the form of acetate.

In the presence of malonate, pyruvate oxidation by liver slices or mince affords acetoacetate in quantities up to 25 per cent of the pyruvate metabolized (33, 34, 96). Pyruvate is quantitatively converted to acetoacetate by the washed liver cell preparation of Lehninger (66). When fumarate was added to the same system, acetoacetate formation was suppressed in favor of an increased yield of di- and tri-carboxylic acids. The *in vitro* conditions which affected the relative yields of ketone bodies and polycarboxylic acids respectively were the same whether octanoic acid or pyruvate served as substrates. On the basis of these findings it would appear that a common oxidation product is formed from both compounds. A view which has been widely accepted is that an acetyl-like two carbon unit forms the intermediate step at which the pathways of carbohydrate and fat metabolism converge.

However, certain difficulties are encountered when an attempt is made to correlate the ketone body formation from pyruvate *in vitro* with the metabolic fate of fat and carbohydrate in the intact organism. It is well known that in the starving or diabetic animal fatty acids enhance ketone body formation, whereas pyruvate or lactate reduce ketosis. Evidently, under these conditions, the two substances do not enter a common path. It is possible that acetoacetate formation from pyruvate is the favored reaction under special conditions *in vitro*, but it may still be quantitatively insignificant for the pyruvate metabolism of the

intact animal. The hypothesis that a C<sub>2</sub> fragment arises in the oxidation of both carbohydrate and fat would seem to be untenable unless it is assumed that the C<sub>2</sub> fragments are not identical.

The contrasting behavior of labeled fatty acids and pyruvate in the acetylation of foreign amines in intact animals leads to similar conclusions (30). While acetate is an effective source of acetyl for both aromatic amines (sulfanilamide) and  $\alpha$ -amino acids (phenylaminobutyric acid), alanine (pyruvic acid) can yield acetyl to foreign  $\alpha$ -amino acids only. Moreover, labeled alanine, unlike acetate or butyrate, does not serve as a precursor for cholesterol, either *in vivo* (30) or *in vitro* (97). If pyruvate were converted to the same C<sub>2</sub> fragment which is active in the acetylation of foreign amines and in cholesterol formation, labeled alanine or pyruvate should show the same effect as acetate. Recent experiments by Sonne et al. (98) on uric acid formation contain similar evidence. The feeding of CH<sub>3</sub>C<sup>13</sup>OOH resulted in the incorporation of heavy carbon at carbon atoms 2 and 8 of the purine nucleus. No significant concentrations of C<sup>13</sup> appeared at these positions when C<sup>13</sup>H<sub>3</sub>C<sup>13</sup>HOHCOOH was fed, indicating that lactate is not broken down to the two carbon compound employed in uric acid synthesis.

It is conceivable that the differences observed are quantitative rather than qualitative, but it is not likely that the bulk of pyruvate is oxidized by way of the same C<sub>2</sub> fragment which arises in the catabolism of fatty acids. Bloch and Rittenberg have concluded that the amounts of acetate produced in the intact rat can be accounted for fully by the oxidative metabolism of fatty acids and keto-genic amino acids (30). These data also would seem to exclude any considerable breakdown of pyruvic acid by way of acetyl as an intermediate.

Acetymethylcarbinol (acetoin) has been obtained as a product of the anaerobic metabolism of pyruvate in micro-organisms (99) and in animal tissues (heart muscle) (100). The process is believed to involve oxidative decarboxylation to acetaldehyde, condensation of the latter with a second molecule of pyruvic acid, and subsequent decarboxylation of the condensation product. A scheme involving the following steps: pyruvate  $\rightarrow$  acetoin  $\rightarrow$  diacetyl  $\rightarrow$  acetyl phosphate  $\rightarrow$  acetate—has been proposed by Doisy and Westerfeld (24) on the basis of the effect shown by acetoin in the acetylation of p-aminobenzoic acid. The relatively high toxicity of acetoin for animals (101) and the fact that it is in part excreted after reduction to butyleneglycol (102) seems to preclude the possibility that the above scheme represents a major pathway of pyruvate metabolism.

The formation of acetate from pyruvate by an indirect route involving the splitting of intermediates of the tricarboxylic acid cycle has become a possibility in view of the following observations. The fermentation of citrate by yeast (103) and by various bacteria (104, 105, 106) has been found to yield 1 to 2 moles of acetate and smaller amounts of formate and succinate. These results have been interpreted to suggest the following series of reactions: citrate  $\rightarrow$  acetate + oxaloacetate  $\rightarrow$  pyruvate + CO<sub>2</sub>  $\rightarrow$  acetate + formate. The first of these steps, i.e., the reversal of the condensation of acetate and oxaloacetate is believed by Lipton and Barron (107) to be responsible for the positive effect shown by citrate in the acetylation of choline in brain extracts. The reversibility of the initial

condensation reaction in the tricarboxylic acid cycle has not been tested in other systems of animal origin.

Slade and Werkmann (108) have studied the conversion of succinate to acetate by *Aerobacter indologenes* with the aid of C<sup>13</sup>. Although these authors conclude that their data indicate a splitting of succinate into two molecules of acetic acid, it is equally possible that the acetate arose not from succinate directly but by degradation of either  $\alpha$ -ketoglutarate or of a tricarboxylic acid formed from succinate. Irrespective of the pathway involved, these results suggest a possible route for the conversion of carbohydrate intermediates to acetate and fatty acids which circumvents the decarboxylation of pyruvate itself.

UTILIZATION OF ACETIC ACID FOR SYNTHETIC REACTIONS. The administration of labeled acetic acid to animals has been found to result in the incorporation of isotope into a variety of tissue constituents: glycogen, cholesterol, fatty acids, the dicarboxylic amino acids, protoporphyrin and uric acid. It thus appears that in the biological formation of the cell constituents acetic acid is of general importance as a source of carbon atoms. It has become increasingly evident in recent years that body constituents of high molecular weight are synthesized by condensation of numerous small sized units rather than by the utilization and rearrangement of preformed large molecules.

In a few cases only the animal organism depends exclusively on an exogenous supply for the maintenance of cellular composition (vitamins, essential amino acids and essential fatty acids). The majority of the tissue constituents can be derived not only from unchanged dietary material but can also arise by synthesis from small molecular breakdown products which are formed in intermediary metabolism. The ability of the animal organism to keep its tissue composition constant under a variety of dietary conditions is an expression of the fact that the required elementary building stones are available from a number of sources.

Older suggestions that cholesterol might be formed either by modification of the preformed steroid structure which is offered to the animal in the form of squalene (109) or plant steroids (110), or by folding of long chain fatty acids (111) have given way to the view that a total synthesis from numerous small units is involved (112). In the synthesis of long chain fatty acids from carbohydrate the sugar molecule is presumably not utilized as such but only after its degradation to C<sub>2</sub> or C<sub>3</sub> fragments. In the formation of the tetra pyrrole structure of porphyrin, two C<sub>2</sub> compounds, acetic acid (30) and glycine (113, 114), serve as a source of carbon and nitrogen and not the preformed five-membered ring structures of proline or pyrrolidonecarboxylic acid (from glutamic acid). The investigations on the biological synthesis of these high-molecular tissue constituents have so far been carried out mainly with intact animals. It is evident that for the synthesis of the more complex molecules a high state of cellular organization will be required. For this reason virtually no attempts have been made to investigate the enzymatic processes concerned in the synthesis of the substances mentioned. It has not been possible to identify any of the intermediate steps in the biosynthesis of fatty acids, cholesterol or porphyrin, nor do indications exist that such intermediates occur normally in measurable quantities.

The utilization of acetic acid for biological syntheses has been demonstrated by the feeding of labeled acetic acid to animals and isotope analysis of the body constituents isolated from the tissues. Insight into the reaction mechanisms has in some cases been gained by determining the location and distribution of isotope at various positions of the synthetic product, but the accessible information is often limited by the lack of suitable methods of degradation. Wood et al. (115) have employed chemical and microbiological degradation procedures for glucose which permit separate isotope analysis of carbon atoms 3 and 6 and of fractions which represent carbon atoms 1 and 6, 2 and 5, and 3 and 4 respectively. Methods suitable for determining the deuterium concentrations at separate positions of deuterio glucose have been described by Stetten and Stetten (116). Although procedures are available for successive degradation of the higher fatty acids they are not applicable to small quantities of biological material. However, analysis of the carboxyl group and of the two moieties obtainable by degradation of oleic acid have yielded valuable information on the mechanism of fatty acid synthesis (117). The distribution of isotope in deuterio cholesterol has been determined by degradation of the steroid molecule into nucleus and side chain (118). No experiments of this type have so far been carried out with protoporphyrin formed biologically in the presence of labeled acetate.

Although it may be feasible to study details of reaction mechanisms by employing test substances which contain more than one isotopic marker, there are certain limitations to the experimentation with intact animals. If, in a synthetic process, acetic acid did not take part as such but in the form of a functional derivative (acetylphosphate, acetylamine) and if the substituent were eliminated in the course of the condensation, isotope analysis of the reaction product may not reveal whether acetic acid reacted as such or in a combined form. On the other hand, experiments with intact animals are indispensable in order to establish the occurrence and the quantitative significance of a process which has been observed in an isolated system.

*Glycogen.* According to classical concepts, a glycogenic compound is a substance which either increases glycogen deposition in the liver of a fasted animal or enhances glycosuria in the diabetic animal. Acetic acid does not satisfy these criteria, since its feeding neither increases glycogen deposition nor glucose excretion (119). On the other hand, isotope experiments have shown that acetic acid can provide carbon atoms for all positions of the glucose molecule (120, 121). No inconsistency need be seen in these findings, if (a) the meaning of the term precursor is more clearly defined and if (b) it is recognized that the terms glycogenic and ketogenic apply only to special experimental conditions. The revision of classical concepts necessitated by the results obtained with tracer substances has been discussed by Buchanan and Hastings (122) and by Wood (123). If one disregards reactions in which a substance effects an increased formation of a product by stimulation rather than by direct conversion, then two types of precursors have to be considered: substances which afford a net increase of the quantity of product, i.e., reactions in which the quantity of precursor is the limiting factor, and secondly substances which are incorporated either totally or

in part into reaction products but which are without effect on the rate of its formation. Compounds which in classical terminology are classified as either glycogenic or ketogenic belong to the first group while acetic acid with respect to glycogen formation belongs to the second category. Although, as Lorber, Lifson and Wood (121) have shown, acetic acid provides carbon atoms for all positions of the glucose molecule, it probably cannot by itself form hexose (or pyruvate) but only by entering the tricarboxylic acid cycle. Through a series of transformations the tricarboxylic acid yields pyruvate, which is the obligatory intermediate in glucose formation. Acetate utilization for glycogen synthesis is therefore not regulated by the availability of acetic acid but depends on tricarboxylic acid formation and regeneration of pyruvate from the latter. On the other hand the conversion of pyruvate to glycogen does not require the intervention of the citric acid cycle and as a result pyruvate and compounds which are directly convertible to pyruvate will be precursors of glycogen in the sense that they can increase the net amount of glycogen formed. It is evident that the terms glycogenic and ketogenic apply only to conditions in which a change of carbohydrate concentration is observable, i.e., in the starving or diabetic animal. From its behavior in isolated liver, pyruvic acid could be classified as a ketogenic substance, since under such conditions it can be converted in part (124, 33, 90, 96) or quantitatively (125) to acetoacetic acid. Acetic acid is ketogenic since it increases ketone body excretion in the starving or diabetic animal (5), but as isotope studies have shown, it is also a source of carbon atoms for glucose. The appearance of acetate carbon in all positions of the glucose molecule has been ascribed by Wood (115) to a series of steps by which a tricarboxylic acid formed from acetate and oxalacetate is degraded to pyruvate by way of  $\alpha$ -ketoglutarate and the C<sub>4</sub> dicarboxylic acids. If C<sup>13</sup>H<sub>3</sub>C<sup>13</sup>OOH is employed the resultant pyruvate will be labeled at all three positions but it cannot contain more than half of the isotope concentration present in the acetate which was utilized. On tracing the labeled carbon through tricarboxylic acid,  $\alpha$ -ketoglutarate and the dicarboxylic acids to succinate, it becomes clear that only two of the four carbons in the dicarboxylic acids originate from acetate but the isotopic carbon becomes evenly distributed over the entire carbon chain when the asymmetry of the molecule is lost at the succinate stage. Pyruvate will therefore contain C<sup>13</sup> at all positions. It can be concluded that although acetate carbon appears in all positions of glucose not more than half of the carbon atoms of a glucose molecule can have originated from acetate, the remainder being supplied by oxaloacetate. Acetate per se is therefore incapable of forming glucose.

Carboxylation of acetic acid to pyruvic acid cannot be an important pathway for the utilization of acetate in glycogen formation because in this case the isotope distribution in glycogen would be expected to differ from that actually observed (121).

*Fatty acids.* McLean and Hoffert (126) have studied lipid formation in yeast from acetate and have concluded from balance data that both steroids and neutral fat could be synthesized directly from acetate without intermediate formation of carbohydrate. In the presence of sulfite steroid synthesis was

inhibited, but fat continued to be synthesized. This suggested that acetaldehyde might be an intermediate in the synthesis of yeast steroids while acetate could condense directly to fatty acids. In their experiments on the metabolism of deuterioacetate in yeast, Sonderhoff and Thomas (127) found the following concentrations of deuterium in the various fractions: carbohydrate 1.6 per cent; fatty acids 14.7 per cent; unsaponifiable 30 per cent, indicating that carbohydrate was not an intermediate in the conversion of acetate to lipids.

Wood et al. (128) have demonstrated the bacterial utilization of acetic acid for the synthesis of lower fatty acids. In the presence of  $\text{CH}_3\text{C}^{13}\text{OOH}$ , the butyl alcohol formed in the fermentation of *Cl. butylicum* and of *Cl. acetobutylicum* was found to contain  $\text{C}^{13}$  at carbon atoms 1 and 3. Since these organisms can reduce butyric acid to butanol, the following series of reactions is suggested: 2 acetate → butyrate → butanol.

The fermentation of alcohol and acetate in *Cl. kluyveri* has been studied by Barker, Kamen and Bornstein (129). The butyric acid formed in the presence of  $\text{CH}_3\text{C}^{14}\text{OOH}$  contained approximately equal quantities of heavy carbon in the carboxyl and  $\beta$  positions; in caproic acid equal amounts of  $\text{C}^{14}$  were presumably present at alternate carbon atoms since the carboxyl group accounted for about one-third of the total isotope content of the hexanoic acid. Important information with respect to the mechanism of fatty acid synthesis in these organisms was obtained by the same authors. Isotopic caproate formed in the presence of  $\text{CH}_3\text{CH}_2\text{CH}_2\text{C}^{14}\text{OOH}$  and normal ethanol contained isotope but this was not present in the carboxyl group. The active  $\text{C}_2$  unit derived from ethanol must therefore have condensed with the carboxyl group of butyric acid. Moreover, a breakdown of butyrate to  $\text{C}_2$  compounds prior to formation of the  $\text{C}_6$  acid cannot have occurred since in this case the carboxyl carbon of caproic acid should have contained the carbon isotope.

Rittenberg and Bloch (117) have fed mice and rats acetic acid which contained  $\text{C}^{13}$  at the carboxyl position and deuterium in the methyl group. The fatty acids isolated from the tissue lipids contained both isotopes in excess of the concentrations which could have resulted from the oxidation of acetate to  $\text{C}^{13}\text{O}_2$  and  $\text{D}_2\text{O}$  and incorporation of deuterium from the body fluids or  $\text{C}^{13}$  by a reaction involving  $\text{CO}_2$  assimilation. The body fluids of the mice contained 0.09 per cent D and the respiratory  $\text{CO}_2$  contained 0.066 per cent  $\text{C}^{13}$  while the saturated fatty acids isolated from the liver lipids contained 0.42 per cent D and 0.16 per cent  $\text{C}^{13}$ . As the fatty acids contained both  $\text{C}^{13}$  and deuterium it is clear that the acetic acid molecule as such had been utilized in the synthetic process. From acetic acid containing isotopic carbon in the carboxyl group, a fatty acid containing  $\text{C}^{13}$  only at one out of two carbon atoms is to be expected. The incorporation of isotope into the fatty acids could result from the following processes: 1, acetic acid elongates the fatty acid chain by adding at the carboxyl end. In this case the presence of isotopic carbon should be confined to the carboxyl carbon of the fatty acid and decarboxylation should leave a non-isotopic residue; 2, If acetic acid condensed with the  $\omega$  carbon of the fatty acid decarboxylation should yield  $\text{CO}_2$  which contains no excess of isotope; 3, acetate carbon is uniformly

distributed over the fatty acid chain.  $C^{13}$  should then be present at the odd-numbered carbon atoms. The  $CO_2$  obtained on decarboxylation of the fatty acid actually contained about twice as high an isotope concentration as the entire molecule, indicating that  $C^{13}$  was present at alternate positions, i.e., at the odd-numbered carbon atoms, one of which is the carbon of the carboxyl group. Additional support for the view that the acetic acid carbon was distributed in random fashion over the entire fatty acid chain is afforded by analysis of the degradation products of the "oleic acid" fraction of tissue fat. This consists mainly of oleic acid and some palmitoleic acid and yields on oxidation a monocarboxylic acid, pelargonic acid, derived from carbon atoms 10 to 18 and a dicarboxylic acid (azelaic acid) representing carbon atoms 1 to 9. If a  $C_{18}$  fatty acid were formed from nine acetate units then of the nine isotopic carbons, five should be present in the azelaic acid and four in the pelargonic acid fraction, while the deuterium content of the two moieties should be nearly equal. The following isotope concentrations were found: azelaic acid, 0.090 per cent  $C^{13}$ , 0.14 per cent D; pelargonic acid, 0.071 per cent  $C^{13}$ , 0.15 per cent D. These data suggest a process of fatty acid synthesis involving the multiple condensation of  $C_2$  units. Any scheme proposed as a mechanism of fatty acid synthesis must account for the fact that the fatty acids of animal tissues have an even number of carbon atoms and comprise, if milk fat is included, all members of the series from  $C_4$  to  $C_{24}$ . The occurrence of  $C_2$  condensation in the synthesis of fatty acids by animal tissues was first demonstrated by the finding of Stetten and Schoenheimer (130) that deuterostearic acid is formed from deuteropalmitic acid by chain elongation.

The reactive two carbon compound undergoing the condensation can be readily formed from acetic acid in the intact animal and in bacterial systems, but its chemical identity has not been determined. Fatty acid synthesis in animal cells has so far not been demonstrated in isolated systems. When rat liver slices were incubated with labeled acetic acid only very small amounts of isotope were incorporated into the fatty acids; significant concentrations of deuterium were found in the liver fatty acids when the buffer medium contained  $D_2O$  (131). From this result it cannot be decided whether the uptake of isotope resulted from synthesis of the carbon chain or whether it was due to hydrogenation of unsaturated fatty acids.

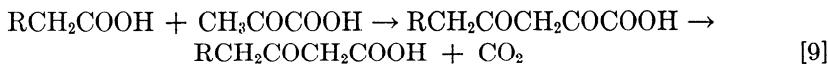
The production of  $C_2$  units in the course of fatty acid oxidation and their utilization in fatty acid formation points to a mechanism of synthesis which consists of the reversal of the catabolic steps. This would involve the condensation of two carbon units with acetate and higher fatty acids and the subsequent reduction of  $\beta$  keto compounds to saturated acids. The well established conversion of acetate to acetoacetate may represent the initial step in this process. However, the biological reduction of  $\beta$ -keto acids to the corresponding saturated fatty acids has never been shown to occur.

Experiments with acetic acid containing deuterium as well as  $C^{13}$  have thrown some light on the nature of the intermediates involved in fatty acid synthesis (117). When a preparation of acetate was fed in which deuterium and carbon

were present in a ratio of 8:1 the fatty acids isolated contained the two isotopes in a ratio of 2.5:1. Irrespective of the nature of the condensation, there should be introduced for every two deuterium atoms from the methyl group of acetic a minimum of two atoms of ordinary hydrogen from the body fluids. The ratio of D:C<sup>13</sup> should then become 4:1. The greater loss of D than corresponds to this ratio may have resulted from keto-enol tautomerism of the intermediate  $\beta$ -keto acid. An additional atom of normal hydrogen from the body fluids would also be introduced if the keto acid were converted to the saturated acid by way of the hydroxy- and unsaturated acids.

Although the isotope data suggest that acetic acid can supply the carbon atoms for all positions of the fatty acid chain, it is unlikely that acetate is a precursor in the sense that its administration produces a net increase of synthesized fat. Experimental conditions for studying fat synthesis from specific precursors, similar to those by which the glycogenic or ketogenic properties of a compound can be tested, are not available. Conditions which result in the deposition of excessive quantities of fat in the liver such as choline deficiency do not measure changes in the amount of fat synthesized but reflect deficiencies in fat transport. Stetten and Boxer found that in rats treated with alloxan, fat deposition and incorporation of deuterium from heavy body water are greatly reduced (132). This may be the result of diminished fat synthesis or of a failure to deposit the newly formed fat. The impairment of fatty acid synthesis in this condition is probably not due to a lack of precursor but may reflect, as Stetten and Boxer suggest, a general inability to utilize carbohydrate. Since in the experiment of Rittenberg and Bloch (117) the animals received a fat free diet, carbohydrate and protein must have provided the carbon for fatty acid synthesis. It was estimated that under these conditions about 25 per cent of the carbon atoms of the fatty acids were derived from acetate. While the conversion of carbohydrate to fat is well established from balance experiments, no information is available concerning the intermediate steps. It is generally assumed that synthesis of the fatty acid chains occurs with pyruvate or a two carbon compound derived from pyruvate. It is questionable whether this intermediate is identical with acetic acid because in this case not only one-fourth but all carbon atoms of the fatty acids should be available from the "acetyl" pool. It has been pointed out above that pyruvic acid or alanine are not convertible to the acetyl compound involved in the acetylation of sulfanilamide or in the formation of cholesterol. This has been taken to mean that a direct degradation of pyruvic acid to a C<sub>2</sub> compound does not occur or that the product is not identical with the acetyl formed by fatty acid oxidation or from acetate itself.<sup>3</sup> In order to allow for the participation of both acetic acid and carbohydrate in fatty acid synthesis, Rittenberg and Bloch (117) have suggested that pyruvate may form acylpyruvic acids and that the decarboxylation to  $\beta$ -keto acids takes place after the condensation.

<sup>3</sup>This conclusion was based on experiments with deuterio alanine. It does not take into account the possibility that pyruvate is converted to acetyl by a circuitous route in which deuterium is lost entirely. Recent experiments of Sonne et al. (98) on uric acid synthesis with test substances containing C<sup>13</sup> similarly indicate that lactate is not converted to acetate.



In this reaction the net effect is the addition of a two carbon unit, without acetic acid appearing as an intermediate. It is also conceivable that an intermediate of the tricarboxylic acid cycle which contains the elements of both acetic and pyruvic acids supplies the C<sub>2</sub> fragments for fatty acid synthesis. For instance, the C<sub>4</sub> dicarboxylic acids which are derived from the condensation product of acetate and oxaloacetate are composed of two C<sub>2</sub> moieties one of which originates from acetate and the other from pyruvate. By passing through a symmetrical stage, randomization would result, and in a utilization of the dicarboxylic acid for fatty acid synthesis an equal chance would exist that the C<sub>2</sub> fragment was contributed by either acetate or pyruvate.

It has been pointed out above that the acetic acid arising in intermediary metabolism appears to be derived primarily from the catabolism of fatty acids and ketogenic amino acids while carbohydrate does not contribute significantly to the acetate pool. It would follow, then, that the acetic acid employed for fatty acid synthesis has its origin in the fatty acids themselves; in other words, the process of acetate utilization merely reflects the continuous molecular regeneration of body fats. Since a large portion of the acetyl fragments arising in fatty acid oxidation is metabolized further and is therefore unavailable for fat resynthesis, carbon has to be provided from another source in order to keep the total quantity of fat constant. The source is evidently carbohydrate. Acetate is therefore not a precursor of fatty acids in the conventional sense and the statement that 25 per cent of the carbon atoms are supplied by acetate merely implies that one out of every four carbon atoms incorporated into a newly synthesized fatty acid molecule arose from the catabolism of the fatty acids themselves.

*Cholesterol.* In their experiments on the utilization of deuterioacetate by yeast Sonderhoff and Thomas found the deuterium concentration in the unsaponifiable fraction to be twice as high as in the fatty acid fraction and twenty times that of the yeast carbohydrate (127); they therefore suggested that in the synthesis of the yeast steroids acetate had been employed directly. A formation of cholesterol in animal tissues by total synthesis from small units was indicated by the findings of Rittenberg and Schoenheimer (112). In cholesterol newly formed by animals whose body fluids were enriched with heavy water roughly half of the hydrogen atoms were derived from the D<sub>2</sub>O of the body fluids. The utilization of various small molecular substances for cholesterol synthesis has been investigated by Bloch and Rittenberg in feeding experiments with labeled compounds (133, 118, 78). The isotope concentration in cholesterol isolated from the tissue lipids was highest when labeled acetic acid was the test substance. Whenever another compound was effective in causing the formation of labeled cholesterol the result could be attributed to intermediary formation of acetate from the test substance (butyrate, ethanol, leucine, valerate, isovalerate). By studying simultaneously cholesterol synthesis and the acetylation of foreign amines it could be shown that the ability of a test substance to cause the formation of

labeled cholesterol was roughly parallel with its ability to furnish labeled acetyl groups. This correlation did not exist in the case of alanine. The significance of this finding has been discussed above. An evaluation of the quantitative rôle of acetic acid as a precursor for the steroid structure led to the conclusion that about half of the hydrogen atoms (and probably half of the carbon atoms) are furnished by acetate. Acetic acid appears to contribute to the formation of the entire steroid molecule. When cholesterol formed biologically from deuterio acetate was degraded, the iso-octyl fragment derived from the cholesterol side chain and the hydrocarbon representing the steroid skeleton contained deuterium in nearly equal concentrations. No information is available as to the intermediate steps of the synthetic process. The higher fatty acids or intermediates of fatty acid metabolism cannot lie on the path of the acetate-sterol conversion, since in feeding experiments with deuterio acetate, newly formed cholesterol contains several times the isotope concentration of the fatty acids; also, in liver slices deuterium is rapidly incorporated into cholesterol when labeled acetate is present, but not into fatty acids (131). The utilization of both carbon atoms of acetic acid is evident from the appearance of both deuterium and C<sup>13</sup> in cholesterol synthesized from CD<sub>3</sub>C<sup>13</sup>OOH (117, 131).

Since the acetate-cholesterol conversion entails essentially the transformation of—CD<sub>2</sub>·CO—into—CD<sub>2</sub>·CH<sub>2</sub>—two normal hydrogen atoms should be incorporated for every two carbon-bound deuterium atoms and in the utilization of CD<sub>3</sub>C<sup>13</sup>OOH the ratio of D to C<sup>13</sup> should decline to half its original value. However, the relative amounts of D and C<sup>13</sup> introduced into cholesterol were roughly the same as in the administered acetate and it would appear therefore that the reaction product contained, perhaps as the result of decarboxylations, more of the methyl carbons than of the carboxyl carbons of acetic acid.<sup>4</sup>

Some indications exist that a reduction may be the initial step in the acetate-cholesterol conversion. McLean and Hoffert (126) observed that sterol synthesis from acetate in yeast was inhibited by sulfite, a reagent which blocks aldehyde groups. In the formation of labeled cholesterol by rat liver slices, labeled ethanol and acetaldehyde were somewhat more effective than acetic acid (97) suggesting that the intermediate is more readily formed from ethanol and acetaldehyde. It is unlikely that the somewhat higher efficiency of acetaldehyde and ethanol under these conditions is due to the fact that they diffuse more readily into the liver cell since the deuterium concentration in cholesterol is independent of the acetate concentration in the buffer medium over a wide range (131).

In the intact animal and in liver slices deuterio alanine fails to give labeled cholesterol (118, 97). Alanine, and presumably pyruvic acid, are therefore not convertible to the C<sub>2</sub> compound which provides carbon for cholesterol synthesis and which is readily formed from acetic acid itself and from the higher

<sup>4</sup> The results obtained with intact animals differ from those obtained in liver slices. The ratio of D to C<sup>13</sup> in cholesterol formed from acetate in vitro changes from 8 to 2.5. This might be explained by the loss of carbon bound deuterium associated with the interconversion of acetate and acetoacetate which proceeds rapidly in liver slices but apparently not in the intact animal.

fatty acids. This finding supports the previously expressed view that if a C<sub>2</sub> fragment arises in carbohydrate oxidation it is unlikely to be identical with that derived from fatty acids.

The origin of the remaining carbon and hydrogen atoms in cholesterol, which are not accounted for by acetic acid, is unknown. In view of the fact that cholesterol contains a branched side chain and angular methyl groups and that these groupings are otherwise present only in dietary constituents which animals cannot synthesize (valine, leucine, carotene), the possibility has been tested that one of the branched chain amino acids participates in steroid synthesis. The feeding of leucine which contained deuterium at all positions of the carbon chain resulted in the formation of labeled cholesterol while similarly labeled valine was ineffective (78). However, leucine is a ketogenic amino acid and in the course of its catabolism yields acetyl groups. Hence, no conclusions may be drawn as to whether leucine specifically supplies the branched side chain and the angular methyl groups of cholesterol or whether the observed effect is ascribable merely to the intermediary formation of acetic acid.

Cholesterol can serve as the parent substance for other compounds which possess the cyclopentano-phenanthrene structure. The *in vivo* transformation of cholesterol into cholic acid (134) and into pregnanediol (135) has been demonstrated with the aid of deuteriocholesterol and it has been found that a minimum of two-thirds of these steroids can arise by degradation of cholesterol. It is therefore not too likely that animal steroids other than cholesterol can also be formed by total synthesis from small units by a pathway in which cholesterol is not an intermediate.

*Protoporphyrin.* Hemin isolated from the red cells of animals which receive deuterio acetic acid contains deuterium in significant concentrations (30). In short term feeding experiments the isotope content of hemin will attain only a relatively low level, since the protoporphyrin moiety of hemoglobin is synthesized at a much slower rate than most body constituents (114). When deuterioacetate feeding is extended to a period of several weeks the deuterium concentration in hemin reaches a value corresponding to 50 per cent of the isotope concentration in the acetic acid available in the metabolic pool (136). Therefore at least half of the hydrogen atoms in the porphyrin molecule are supplied by acetic acid. The constituent pyrrol nuclei in the porphyrin molecule contain no hydrogen linked to carbon; carbon bound hydrogen is present in the methin bridges which link the pyrrol rings and in the pyrrol side chains. Hence, experiments with deuterio acetic acid do not reveal whether acetic acid is also a source of carbon atoms for the pyrrol rings. A mechanism of synthesis which leads directly to a pyrrol structure containing the appropriate side chains would seem to be a more likely event than a process in which the substituent side chains are attached to a pre-formed pyrrol nucleus. No deuterium is introduced into hemin after the feeding of deuterio propionic acid while  $\beta$ - $\gamma$ -dideuterio butyric acid is about half as effective as deuterio acetic acid (136). The effect shown by butyrate is most likely due to its splitting into acetyl fragments.

The origin of pyrrol nitrogen has been elucidated by the experiments of

Shemin and Rittenberg (114, 137). Hemin isolated from the blood of a human subject who had received glycine labeled by N<sup>15</sup> contained so high an isotope concentration as to indicate a specific utilization of glycine nitrogen. The specific role of glycine in pyrrol synthesis received further support from the finding that glycine was the most effective source of pyrrol nitrogen among several amino acids tested (114). In particular, proline and glutamic acid, which have been suggested as possible biological precursors of pyrrols because they contain or can readily form heterocycles similar to pyrrols, were much less effective as sources of nitrogen for hemin than glycine. The utilization of the two carbon compounds, acetic acid and glycine, points to a mechanism of porphyrin synthesis in which the ring structure and the substituents are formed simultaneously, possibly by processes analogous to those which yield pyrrols in organic chemistry. Fischer and Fink (138) have obtained evidence that ring closure to a pyrrol occurs when glycine and formylacetone (the aldehyde of acetoacetic acid) react in aqueous solution at 37°. The classical pyrrol synthesis of Knorr (139) involved the condensation of α-amino acetoacetic ester with acetylacetone. Acetic acid can be converted to aceto-acetate in vivo and it is not inconceivable that by an analogous process α-amino-acetoacetic acid is formed biologically from acetic acid and glycine. A biological pyrrol synthesis analogous to the Knorr reaction would be attractive because it would afford a product in which the arrangement and the chemical nature of the substituents resembles that in the naturally occurring protoporphyrin.

*Uric acid.* Recently the utilization of C<sub>2</sub> compounds for the synthesis of still another group of tissue constituents has been reported. Sonne, Buchanan and Delluva (98) have studied the incorporation of C<sup>13</sup> into uric acid, isolated from pigeon excreta, after the feeding of CH<sub>3</sub>C<sup>13</sup>OOH, CH<sub>3</sub><sup>13</sup>C<sup>13</sup>HOHCOOH, CH<sub>3</sub>CHOHC<sup>13</sup>OOH and NH<sub>2</sub>CH<sub>2</sub>C<sup>13</sup>OOH respectively. The carboxyl carbon of acetic acid was located at the two ureido carbons (2 and 8) of the purine structure, while the carboxyl group of glycine supplied carbon for position 4, and CO<sub>2</sub> carbon for position 6. On the basis of the data available so far no plausible mechanism for purine synthesis from CO<sub>2</sub>, acetate and glycine can be suggested. It is of interest in this connection that Barker and Beck (140) found ammonia, carbon dioxide, acetic acid and small amounts of glycine to be formed as products of uric acid fermentation by *Clostridium acidi urici*. The findings of Sonne et al. contain important evidence with respect to some other metabolic processes. The appearance of acetate and glycine carbon at different positions of the uric acid eliminates the possibility that in the pigeon glycine and acetic acid are interconvertible. On the basis of nutritional studies, Almquist et al. had contended that the chick is unable to synthesize adequate amounts of glycine and that in deficient diets acetate may serve as substitute for glycine (141). The interconvertibility of the two compounds which is implied by these findings is contradicted by the tracer experiments of Sonne et al. unless metabolic differences exist between the chick and the pigeon.

The isotope data obtained by Sonne et al. furthermore show that the α carbon of lactate is not incorporated into the same positions of uric acid as the carboxyl

carbon of acetate. A breakdown of lactate to acetate did, therefore, not occur to any considerable extent. These results are incompatible with the assumption that the hypothetical two carbon compound arising in pyruvate oxidation is closely related to acetic acid.

A relationship between acetate and lipid metabolism in bacteria has been observed by Guirard, Snell and Williams (142). Acetate stimulates the early growth of lactic acid bacteria, an effect which is also shown by fatty acids and various steroids. These results suggest an important rôle of acetate in the synthesis of lipids by bacterial systems.

As the result of investigations with isotopically labeled test substances there have been developed a number of novel biochemical theories among which Schoenheimer's concept of the dynamic state of the body constituents has been the most outstanding (143). According to this concept, there exists a pool of metabolites which is continuously replenished by the breakdown products of both dietary and tissue constituents and which supplies the metabolites for energy production and for the rebuilding of the structural components of the cell. The concept of the metabolic pool was advanced in order to interpret the data obtained from feeding experiments with isotopic substances and in order to provide a reasonable basis for the rapid interchange of dietary and tissue elements which was indicated by these results. It should be pointed out that the metabolic pool may not possess any physical reality in the sense that there exists in the body at a given moment a large reservoir of metabolites in which molecules derived from dietary and various tissue constituents are distributed in random fashion and are therefore undistinguishable. It is merely permissible to say that if for example 100 molecules of dietary origin enter the tissues during a given period of time and 100 molecules arise from body constituents during the same interval, then the chances that in a subsequent reaction either the exogenous or endogenous molecule is employed will be equal. The metabolic pool should therefore be regarded merely as an expression of this probability.

One of the problems of metabolism which has held the general interest of the biochemist has been the mutual interconversion of fat, carbohydrate and protein. These questions have only partially been answered by balance techniques in which the criterion of a biochemical conversion is the net change in amount of the reaction product which is induced by the precursor. In the light of the concept of the metabolic pool, biochemical interconversions assume a different meaning. Fat, protein and carbohydrate are interconvertible in the sense that they give rise to breakdown products which lose the identity of their origin and can be utilized interchangeably for the resynthesis of either one of the three major tissue constituents.

The fact that acetic acid is employed as a building stone in the synthesis of a variety of compounds of different chemical structure and function, viz. lipids, carbohydrate, porphyrin, uric acid and some of the amino acids, suggests its direct or indirect participation in the biological formation of all compounds which can be synthesized by the animal cell. If this is true then acetic acid cannot be regarded as a specific precursor of a particular tissue constituent, but its utiliza-

tion would reflect merely the fact that the body employs two carbon units as a principal source for synthetic reactions.

*The biologically active form of acetic acid.* Since in isolated tissues acetic acid shows less reactivity than in the intact animal the possibility has been widely discussed that acetic acid is metabolically converted into a more reactive form. In organic chemistry such activations are achieved by esterification or anhydride formation (acetylchloride, acetic anhydride, ketene, ethylacetate).<sup>5</sup>

Experimental evidence for the biological formation and the participation in a synthetic reaction of an acetic acid derivative rather than of acetic acid itself has been provided in two instances. In bacterial systems the phosphoroclastic splitting of pyruvate to acetylphosphate and formate can be reversed (145). No other function for acetylphosphate has been found so far. A process of intermolecular acetyl transfer in which acetyl groups are shifted from an N-acetyl amino acid to a free amino acid has been indicated by acetylation studies in the intact animal (35).

There are two other well known biochemical processes which are classified as transfer reactions, transfer of methyl groups involving choline, methionine and creatine (146) and the amidine transfer from arginine to glycine in the formation of guanido acetic acid (147, 148). Such reactions cannot be described in terms of ordinary chemical equations involving stable compounds as intermediates. It is conceivable that group or radical transfer may be a more general phenomenon in biological systems and that the answer to the acetyl problem may be sought in this direction.

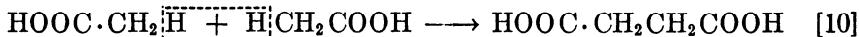
**THE OXIDATIVE METABOLISM OF ACETIC ACID.** The ability of animal tissues to utilize acetic acid efficiently is illustrated by the fact that when fed to animals acetic acid does not appear in the urine in measurable amounts. In the experiments of Buchanan, Hastings and Nesbett with acetate containing radioactive carbon in the carboxyl group, over fifty per cent of the acetate absorbed by the rats was totally oxidized within two hours, as determined by the appearance of isotopic carbon in the expired carbon dioxide (120). This rate was comparable to that at which similar quantities of lactic acid were metabolized (149).

Acetate disappears aerobically from a variety of isolated tissues, the rate being most rapid in the cortex of guinea pig-kidney (150). Little evidence exists that the acetate molecule as such can undergo oxidative reactions.<sup>6</sup> Products which could conceivably arise by oxidation of the methyl group of acetic acid such

<sup>5</sup> For a consideration of the "active" form of acetic acid the following organic chemical reactions are of interest. The studies by Bergmann et al. (36) on intermolecular transfer of acetyl groups or radicals between amino compounds have been mentioned above. Kharasch and Gladstone (144) have shown that by conversion into diacetyl peroxide, the methyl groups of acetic acid become sufficiently activated to undergo  $\alpha$ - $\alpha$  condensation to succinic acid. The reaction is believed to be due to the dimerization of an intermediate  $\text{CH}_2\text{COOH}$  radical.

<sup>6</sup> The claim of Toenniessen and Brinkmann (151) that formate is a product of acetate metabolism in perfused muscle is based on insufficient experimental evidence, but conforms with recent observations of Buchanan and Sonne (167) on the rôle of acetate and formate respectively as precursors of uric acid.

as glycolic, glyoxylic, oxalic acids or glycine are metabolically inert under conditions which permit of rapid oxidation of acetate (152). Many years ago Thunberg suggested that the oxidation of acetate was initiated by the dehydrogenative coupling of the methyl groups of two molecules of acetate to succinic acid (55).



The same reaction was proposed later by Wieland and Sonderhoff to account for the formation of succinate by yeast in which acetate was the sole nutrient (153). Slade and Werkmann (108) have studied this process with C<sup>13</sup> containing acetate in *Aerobacter indologenes* and have concluded that the isotope data demonstrate methyl condensation of two moles of acetate. Lipmann has suggested that this condensation may involve acetylphosphate (154). The inhibition by malonate of acetate and acetoacetate disappearance in animal tissues (155, 156) and of acetate in yeast (157) has been cited in support of reaction [10] but the fact that acetate oxidation is sensitive to malonate may merely be taken to suggest that succinic acid lies on the path of acetate metabolism; it does not indicate the mechanism involved. There is increasing evidence that the step which precedes acetate oxidation consists of a condensation with a C<sub>4</sub> dicarboxylic acid. From the resultant C<sub>6</sub> tricarboxylic acid two carbon atoms are removed by oxidation to regenerate the dicarboxylic acid:



The experimental support for this scheme coincides in many instances with the evidence which led originally to the formulation of the "citric acid" cycle for the metabolism of pyruvic acid. In fact, in some respects the rôle of the tricarboxylic acids is better understood for the oxidative phase of fatty acid and acetate metabolism than for the oxidation of pyruvate. Acetate and acetoacetate have been shown to form the intermediate polycarboxylic acids of the cycle, but it has remained undetermined whether pyruvate itself enters into combination with the C<sub>4</sub> dicarboxylic acids or whether the condensation is preceded by a degradation of pyruvate to a two carbon unit. The significance of the citric acid cycle in the metabolism of fat and carbohydrate respectively is still a matter of controversy. According to Breusch, carbohydrate is not oxidized to an appreciable extent by way of the citric acid cycle (158), while Weil-Malherbe (159) and Krebs (160) reject the view that the citric acid cycle is involved in the oxidation of acetoacetate.

Acetoacetate, acetate and pyruvate have been shown to be convertible, in the presence of dicarboxylic acids, to citrate and intermediates of the tricarboxylic acid cycle, but whether a single mechanism is invoked in all cases is undecided. However, the citric acid cycle constitutes the most successful attempt to date to describe the oxidative phases of fat, carbohydrate and protein metabolism by a unified scheme which visualizes a junction of metabolic pathways and leads to a common mechanism for the ultimate oxidation of the three major dietary and tissue constituents.

The citric acid cycle in relation to the metabolism of carbohydrate has been discussed in reviews by Werkmann and Wood (161), Krebs (162) and Evans (163). The citric acid cycle as a vehicle for fat oxidation has been considered in detail by Wood (123). The present discussion will be concerned primarily with experimental data which deal with the oxidation of acetic acid.

Since there is little evidence to support the assumption that the carbon chain of acetate as such is susceptible to oxidation the question arises as to the steps which precede the conversion of acetate to carbon dioxide and water. Breusch (158) has suggested that the intermediate  $\beta$  keto acids formed in fatty acid oxidation combine directly with oxalacetate to give a product which contains the two terminal carbon atoms of the fatty acid chain, leaving a fatty acid shortened by two carbon atoms. According to this view, two carbon compounds would not arise as distinct intermediates in fat oxidation. Since acetic acid is a compound of great chemical stability and, compared to other metabolites, relatively unreactive in some biological systems, the view has frequently been expressed (123) (164) that acetic acid itself may never arise but may be the stabilization product of a more reactive C<sub>2</sub> unit. The identity of this intermediate has not been established in animal tissues. Judging from the variety of reactions which it undergoes in the intact animal and in some isolated systems, acetic acid must be readily convertible to the hypothetical reactive C<sub>2</sub> fragment.

*C<sub>2</sub> + C<sub>1</sub> addition.* The reversal of the phosphoroclastic splitting of pyruvic acid to formate and acetylphosphate according to the equation:



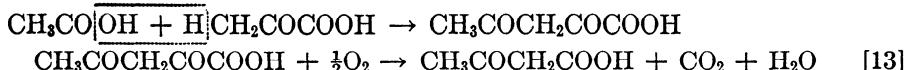
is well established in bacterial systems (145). Lipmann has demonstrated convincingly that acetyl phosphate is the product of this bacterial dissimilation (165). The preservation of a large fraction of energy in the carboxylphosphate facilitates the reverse reaction, i.e., the carboxylation of the C<sub>2</sub> compound, a reaction which is energetically improbable with acetate itself. It has not been possible to demonstrate acetylphosphate as a product of pyruvate metabolism in animal tissues nor can the evidence for the formation of acetylphosphate from acetate in animal tissues be considered sufficient. Lipmann and Tuttle (166) have incubated extracts from pigeon liver with acetate and adenosine triphosphate and observed that on addition of hydroxylamine and ferric chloride a chromogen was formed which had the properties of hydroxamic acids obtainable from acetylphosphate or aldehydes, but more direct proof is desirable to establish the identity of the acetyl compound formed under these conditions. The reversal of reaction [12] is a formylation rather than a carboxylation and apparently CO<sub>2</sub> can be utilized only in organisms which hydrogenate carbon dioxide to formate. In considering the likelihood that reaction [12] occurs in animal tissues, it should be noted that so far no function has been assigned to formic acid in animal metabolism.<sup>7</sup> Experiments of Lorber et al. (121) in which the isotope distribu-

<sup>7</sup> This statement may have to be revised in view of the recent finding by Buchanan and Sonne (167) that formate is utilized in uric acid synthesis by pigeons. The authors suggest that formic acid may arise by oxidation of acetate.

tion in glucose was studied after feeding of labelled acetic acid contain no evidence for  $C_2 + C_1$  addition. Carboxylation of acetic acid which contained  $C^{13}$  in the carboxyl group would yield pyruvate with the labeled carbon at the  $\alpha$  carbon atom. If such pyruvate were further carboxylated to oxaloacetate and reformed after passing through the stage of a symmetrical dicarboxylic acid, the isotopic carbon would become equalized between the  $\alpha$  and  $\beta$  positions of pyruvate. Glucose formed from the singly labeled pyruvate should contain isotope at positions 2 and 5 and, if formed from doubly labeled pyruvate, in positions 1 and 6 as well. Actually a significant excess of  $C^{13}$  was encountered only at positions 3 and 4. Thus a carboxylation of acetate to pyruvate cannot be responsible for the incorporation of acetate carbon into the glucose units of glycogen. Data pertaining to the formation of glycogen or intermediates of the citric acid cycle in the presence of labeled  $CO_2$  do not permit any deductions as to the occurrence of acetate carboxylation because any labeled carbon entering by the latter reaction becomes indistinguishable from the carbon which is incorporated into the  $C_4$  dicarboxylic acids by the primary carboxylation of pyruvate to oxalacetate.

$C_2 + C_2$  addition. There exist several observations which are consistent with Thunberg's suggestion (55) that succinate might arise by the linking of the methyl groups of two moles of acetate. The conversion of acetate to succinate by yeast with acetate as the sole nutrient was attributed by Wieland and Sonderhoff to the dehydrogenative coupling of acetate (153). In an analogous experiment with deuterio acetate Sonderhoff and Thomas (127) found high concentrations of deuterium in succinate and citrate and interpreted their results as evidence for reaction [10]. The proof for this contention would be conclusive only if the succinate contained close to four atoms of deuterium instead of the two atoms actually found. More than one pathway can account for the observed level of isotope in succinic acid. The same qualification applies to the findings on succinate formation from labeled carbon containing acetate by *Aerobacter indologenes* (108). The possibility that in both cases succinate arose by way of the tricarboxylic acid cycle cannot be excluded. At the present moment no experiments are available which establish the occurrence of reaction [10] in the conversion of acetate to succinate. Whenever succinate formation from acetate is demonstrable, succinate is not the sole product but  $\alpha$ -ketoglutarate and tricarboxylic acids also accumulate. If acetate were metabolized according to equation [10] a mechanism should also be available for the subsequent conversion of succinate to ketoglutarate and citrate. The discovery of the carboxylation of  $\alpha$ -ketoglutarate to oxalosuccinate by Ochoa (168) provides a pathway to the tricarboxylic acids, but the reversibility of the oxidative decarboxylation of ketoglutarate to succinate remains to be demonstrated.

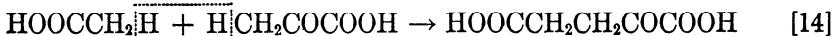
$C_2 + C_3$  addition. The possibilities have been considered that acetic and pyruvic acids combine to form the five carbon acids  $\alpha$ -ketoglutaric and aceto-pyruvic acids. As a result of the current prominence of the tricarboxylic acid cycle these two processes, which would lead directly to five carbon acids, have received diminishing attention. Krebs and Johnson (169) have suggested the formation of aceto-pyruvate according to the equation



as a conceivable intermediate for the conversion of pyruvate to acetoacetate in liver. The utilization of acetopyruvate by intact animals and in isolated tissues has been studied by Lehninger (170). The compound was found to be ketogenic. Since acetoacetate can be metabolized by way of the tricarboxylic acid cycle the reaction offers a chemically plausible scheme for the conversion of pyruvate to the oxidation level of acetate.

Acetopyruvate cannot be an obligatory intermediate in ketone body formation from fatty acids. Weinhause et al. (76), in a study of the oxidation of labeled octanoic acid by liver, found an isotope distribution in acetoacetate which eliminated the participation of pyruvate as an essential component. It is however conceivable that ketone bodies can be formed by two independent synthetic processes, by condensation of two molecules of acetate and by combination of acetate with pyruvate.

$\alpha$ -Ketoglutarate is a key substance in the tricarboxylic acid cycle and a demonstrable intermediate whenever fatty acids or pyruvate are oxidized. A process leading to  $\alpha$ -ketoglutarate by "end to end" condensation of acetate and pyruvate has been suggested by Krebs (171):



Weinhause et al. have observed the formation of labeled  $\alpha$ -ketoglutarate from isotopic acetate and normal oxaloacetate in rat kidney slices or mince, but found no evidence for the simultaneous formation of marked tricarboxylic acids (172). In their experiments non-isotopic citrate and cis-aconitate were added as carriers to facilitate the recovery of citrate at the end of the incubation period. The failure of acetate carbon to become incorporated into citrate in spite of the presence of aconitase, led the authors to the conclusion that citric acid as such was not in the pathway of acetate oxidation by kidney. These results would seem to suggest that  $\alpha$ -ketoglutarate arose from acetate and pyruvate according to equation [14] without intervention of the tricarboxylic acids. However in this case the data exclude rigidly only the formation of citric acid itself. It does not appear to be feasible to isolate isocitric acid or cis-aconitate from such experiments. That the latter two tricarboxylic acids may have been intermediates becomes clear from kinetic data of Martius (173) and Krebs (174). These authors have shown that in systems containing the tricarboxylic acids and aconitase the conversion of cis-aconitate to isocitrate proceeds much more rapidly than to citrate. As the conversion of isocitrate to  $\alpha$ -ketoglutarate is also a rapid reaction it is possible that in the experiments of Weinhause et al. cis-aconitate or isocitrate were the initial reaction products but that the breakdown to  $\alpha$ -ketoglutarate proceeded at a much faster rate than isomerization to citrate.

In a more recent investigation (175) the same authors report that after incubation of kidney with isotopic acetoacetate and normal oxaloacetate, without addition of non-isotopic carrier, citrate could be isolated directly and shown to

contain an isotope level which indicated that about two-thirds of the citrate arose from the isotopic acetoacetate. These recent experiments differed from earlier ones only in that acetoacetate instead of acetate was the substrate. In view of the fact that acetate is readily convertible to acetoacetate under these conditions it is not apparent why labeled citrate should not have formed in both instances.

In an analysis of the available data pertaining to the citric acid cycle, Wood (123) points out that  $\alpha$ -ketoglutarate formation according to reaction [14] as opposed to its formation from tricarboxylic acids cannot be ruled out at the present. If reaction [14] were responsible for  $\alpha$ -ketoglutarate formation rather than the oxidative decarboxylation of isocitric acid, then the formation of tricarboxylic acids would have to be ascribed to the carboxylation of  $\alpha$ -ketoglutarate to oxaloacetate and reduction of the latter to isocitrate. Experiments with labeled test substances and studies of isotope distribution in the  $\alpha$ -ketoglutarate formed from either labeled acetate or acetoacetate have been incapable of providing an answer as to which reaction occurs. Labeled carbon will occupy the same positions whether  $\alpha$ -ketoglutarate is formed by oxidative decarboxylation of isocitrate, directly from acetate and pyruvate or by carboxylation of succinate formed according to equation [10]. The condensation of oxaloacetate and acetate to a tricarboxylic acid does not as such involve an oxidation but can be formulated either as an addition if isocitrate is the primary product, or as a condensation with elimination of water if cis-aconitate is initially formed. It differs in this respect from reactions [10] and [14] in which the coupling process is oxidative. Experimentally it has been found that the formation of the intermediates of the citric acid cycle requires aerobiosis (176) unless a hydrogen acceptor such as oxaloacetate is present in excess (177). This may indicate the occurrence of oxidative condensations or, as is generally believed, oxygen may be necessary to effect the transformation of the reactants into activated forms or derivatives.

There is only scant information on the mechanisms by which carbon-carbon bonds are established biologically. It may be pointed out that in the few processes in which the chemical identity of the reacting molecules seems well established, e.g., in the formation of hexose from triose, or of acetoacetate from acetate, or in the carboxylation of pyruvate to oxaloacetate, at least one of the reacting groups is a carbonyl group. No instances are known of a formation of carbon to carbon bonds by elimination of hydrogen between methyl- or methylene groups.

*C<sub>2</sub> + C<sub>4</sub> condensation.* At present the ultimate oxidation of the split products of fatty acids can be explained most satisfactorily by assuming the intermediate formation of a tricarboxylic acid according to equation [11] and the subsequent breakdown of the condensation product by way of  $\alpha$ -ketoglutaric acid and the C<sub>4</sub> dicarboxylic acids. Reaction [11] was originally proposed by Virtanen (178) to account for citrate formation from acetate in micro-organisms. The following evidence has been secured in support of the suggested rôle of the citric acid cycle for fat and acetate oxidation in various biological systems. Wieland and Sonderhoff (153) found succinate and citrate to accumulate as products of acetate

oxidation by yeast when acetate was the sole nutrient. Under these conditions, the yield of citrate is increased by the addition of oxaloacetate (179). Succinate and citrate formed from deuterioacetate contained 50 per cent and 46 per cent respectively of the deuterium concentration of acetate in stably bound form (127). Lynen has contributed important evidence to show that the formation of succinate from acetate in yeast involves the tricarboxylic acid cycle (157). He demonstrated that the malonate inhibition of acetate disappearance could be overcome by fumarate or oxalacetate and cites this finding as evidence for a requisite participation of the C<sub>4</sub> dicarboxylic acids in acetate oxidation. The observation that the rapid disappearance of acetic acid in yeast is preceded by an induction period was interpreted to show that a second component must accumulate before acetate can be metabolized at the maximum rate. A stimulatory effect of citrate,  $\alpha$ -ketoglutarate and the C<sub>4</sub> dicarboxylic acids has been observed for the aerobic disappearance of acetoacetate in kidney homogenates by Buchanan et al. (180). Wieland and Rosenthal (176) obtained optimum yields of citrate in kidney brei when acetoacetate was oxidized in the presence of oxaloacetate. The identity of the reaction product with citric acid was established by direct isolation. Hunter and Leloir (177) have shown that two molecules of extra citric acid arise from each mole of acetoacetate. They have made the observation that in their experimental system, which consisted of the insoluble particles of kidney cortex homogenate, the simultaneous oxidation of  $\alpha$ -ketoglutarate was essential for citrate formation. This oxidative process is believed to furnish the necessary energy for the conversion of acetoacetate to the intermediate which condenses with oxaloacetate. Lehninger (89) has found that the oxidation of octanoic acid in a preparation of washed rat liver cells yields acetoacetate quantitatively, but that in the presence of fumarate, citrate and  $\alpha$ -ketoglutarate accumulate in significant quantities at the expense of acetoacetate. According to Breusch (181) an enzyme can be extracted from kidney, brain and skeletal muscle which catalyzes the anaerobic condensation of  $\beta$ -keto acids with oxaloacetate to citrate.

In addition to the balance studies a number of experiments have been carried out with isotopically labeled substances. Buchanan et al. (180) incubated kidney homogenates with CH<sub>3</sub>C<sup>13</sup>OOH or acetoacetate marked by heavy carbon at both carboxyl and carbonyl positions. Non-isotopic ketoglutaric acid, fumarate or succinate were added as carriers; when re-isolated after incubation these acids were shown to contain C<sup>13</sup> in the carboxyl positions. It was calculated that the  $\gamma$ -carboxyl of  $\alpha$ -ketoglutarate contained almost ten times as much C<sup>13</sup> as the carboxyl carbon adjacent to the carbonyl group. Essentially the same findings were obtained by Weinhause et al. (172). The formation of labeled citrate from acetoacetate recently demonstrated by the same authors (175) has been mentioned above.

Intermediates of the citric acid cycle can be obtained from isolated tissues on incubation with a variety of substrates but since they are short-lived intermediates *in vivo* they are not obtainable as such in sufficient amounts from intact animals. It has therefore not been possible to secure direct evidence as to

whether the tricarboxylic acid cycle is concerned in fatty acid oxidation also in the intact animal. Feeding experiments have been carried out by Buchanan, Hastings and Nesbett (120) with acetic, propionic and butyric acids, all of which contained C<sup>14</sup> in the carboxyl group. Glycogen isolated from such animals contained radioactive carbon in concentrations which in the case of acetate feeding were attributable to incorporation of CO<sub>2</sub>, but were significantly higher with radioactive propionate and butyrate. Evidently the latter two acids had furnished carbon for glycogen synthesis by conversion to intermediates of carbohydrate metabolism. With the aid of degradation procedures which allow isotope determinations at individual carbon atoms of the glucose molecule (115), Wood and his collaborators have been able to show that the carboxyl carbons of acetic acid appear at positions 3 and 4 (121) and the methyl carbons at positions 1, 2, 5 and 6 of the glucose molecule (182). Thus, both carbon atoms of acetic acid are involved in glycogen formation. Butyric acid CH<sub>3</sub>C<sup>14</sup>H<sub>2</sub>CH<sub>2</sub>C<sup>14</sup>OOH afforded the same isotope distribution in glycogen as CH<sub>3</sub>C<sup>14</sup>OOH (182). From these findings the conclusion can be drawn that there exists a pathway for the conversion of the two carbon split products of fatty acids into the glucose units of glycogen. In this process each carbon atom of glucose can originate from fatty acid carbon.

Glutamic and aspartic acids are believed to be in biological equilibrium with α-ketoglutarate and oxaloacetic acid. Any incorporation of marked fatty acid carbon into intermediates of the tricarboxylic acid cycle should therefore become manifest by the formation of labeled aspartic and glutamic acids. The two amino acids have been isolated by Rittenberg and Bloch from the tissue proteins of mice which had received CH<sub>3</sub>C<sup>14</sup>OOH, and were shown to contain significant concentrations of C<sup>14</sup> (183).

While the evidence which has accumulated in favor of the tricarboxylic acid cycle is impressive it should be emphasized that this scheme does not offer a unique explanation for the formation of tri- and dicarboxylic acids as products of fatty acid oxidation. The following points of the cycle need further clarification: 1, the identity of the intermediate of fatty acid and pyruvate metabolism which condenses with oxaloacetate; 2, the nature of the initial condensation product; 3, the existence of one or more mechanisms for the oxidation of acetate, acetoacetate and pyruvate respectively.

Although the stimulatory effect of the C<sub>4</sub> dicarboxylic acids on citrate formation is exhibited not only by oxaloacetate but also by succinate, malate and fumarate, it is reasonable to assume that the effect is attributable in all cases to oxaloacetate. The nature of the acetyl compound which participates in the condensation process is less evident. In some systems intermediates of the cycle are formed from acetate as well as from acetoacetate, while in other situations the two substances cannot be employed interchangeably. In balance studies with kidney preparations (176, 177) and in the experiments of Lehninger (66) with a heart muscle preparation only acetoacetate was an effective precursor of citrate. The failure of acetate to react in kidney preparations is unexpected because acetate is readily converted to acetoacetate in this organ. On the other hand,

Buchanan et al. (180) found that labeled acetate was as effective as acetoacetate in forming labeled  $\alpha$ -ketoglutarate in kidney homogenate. It should be re-emphasized in this connection that with the aid of the tracer technique a conversion can be demonstrated even if there is no net change in the concentration of the reaction product, e.g., citrate or  $\alpha$ -ketoglutarate. This is important in systems in which the product is metabolized further at a rapid rate.

In general, acetoacetate appears to be the more reactive molecule with regard to the condensation reaction. On the basis of their data Wieland and Rosenthal (176) postulated that acetoacetate itself condensed to yield the hypothetical proctric acids, citroylacetic or acetylcitric acids. Weinhouse et al. were able to isolate C<sup>13</sup> containing citrate from kidney homogenate when acetoacetate was the substrate (175), while in earlier experiments under similar conditions, labeled acetate yielded isotopic  $\alpha$ -ketoglutarate but not citrate (172). The preferential utilization of acetoacetate might be taken to indicate that acetate enters the tricarboxylic acid cycle by way of acetoacetate. However, several situations exist for which this explanation does not hold. Citrate is a product of acetate oxidation in yeast, but as far as is known, the conversion of acetate to acetoacetate does not occur in this organism. Medes et al. have shown that acetate undergoes complete oxidation in heart muscle but that acetoacetate is not an intermediate (86). In kidney also a portion of acetate was oxidized by a route which did not pass through the acetoacetate stage. The data allow for two possible explanations: 1, acetate and acetoacetate enter the tricarboxylic acid cycle by a different process, and 2, a reactive acetyl compound is formed from both acetate and acetoacetate before condensation occurs. In the utilization of acetoacetate a preliminary splitting to acetic acid can be excluded as an intermediate step. Buchanan et al. (184) added normal acetate and isotopic acetoacetate to an extract of rabbit kidney and found that the acetic acid recovered from the mixture after incubation contained appreciably less isotope than  $\alpha$ -ketoglutarate or succinate. From energy considerations it is to be expected that an energy rich acetyl can arise far more readily from acetoacetate than from acetate. The inability of acetate to substitute for acetoacetate may be ascribed to an impairment of the enzyme system responsible for the conversion of acetate to the metabolically active form. Although acetoacetate is an efficient source of citrate in various tissue systems, it is highly doubtful that it is an obligatory intermediate in tricarboxylic acid formation from fatty acids. In the experiments of Lehninger with washed liver cells, acetoacetate, unlike octanoic acid, was incapable of yielding citrate (89). The preparation apparently had lost the property of converting acetoacetate into the active two carbon fragment. The reasons which led to the belief that in the intact animal acetoacetate is not an intermediate of fatty acid oxidation have been discussed above.

Since the experimental conditions employed by various investigators for the study of the same problem vary widely, particularly with respect to methods of tissue preparations, it is not surprising that such experiments have often yielded contradictory results and that it is not possible to fit all available data into a single scheme. It is evident that with increasing impairment of the cellular

organization the enzymatic complement of the biological system becomes increasingly deficient and hence the probability will diminish that a complex series of reactions can take place. Processes which have high energy demands and must be coupled with energy yielding reactions such as the conversion of acetate to an active acetyl are less likely to occur in homogenates or tissue extracts than in systems in which the cell structure is preserved.

Lipmann has shown that by the addition of adenosine triphosphate acetate can be activated to acetyl sulfate anaerobically in pigeon liver extracts (2). The expectations, based on this finding, that the active C<sub>2</sub> compound which is involved in the tricarboxylic acid cycle may be identical with acetyl phosphate, have not been fulfilled. Under conditions in which citrate formation can be demonstrated to occur with acetoacetate but not from acetate, acetylphosphate is also ineffective (184, 177, 89). In order to determine the identity of the active two carbon compound, Lehninger (89) has also tested the effect of glycolate, glyoxalate, oxalate, acetaldehyde, ethanol, acetamide and glycine. None of the compounds yielded extra citrate under conditions which afforded citrate from octanoic acid.

Since *cis*-aconitate, isocitrate and citrate are biologically interconvertible, the identity of the tricarboxylic acid initially formed in the citric acid cycle has remained in doubt. In the presence of aconitase the system will contain all three acids irrespective of the nature of the primary condensation product (173, 185). The cycle as originally proposed by Krebs and Johnson (185), in which citric acid was the initial condensation product, had to be modified when it was found by Evans and Slotin (186) and by Wood et al. (187) that the  $\alpha$ -ketoglutarate formed in pigeon liver homogenate from labelled CO<sub>2</sub> and pyruvate contained labeled carbon only in the carboxyl group adjacent to the carbonyl group, and that when malonate was present, the succinate formed contained little C<sup>13</sup> (186). The tricarboxylic acids could therefore not have originated from a symmetrical citric acid. An unsymmetrical molecule must also have been the precursor of the dicarboxylic acids which Buchanan et al. (180) and Weinhouse et al. (175) obtained as the reaction product of labeled acetoacetate or acetate, and oxaloacetate. In the  $\alpha$  ketoglutarate obtained from the latter experiments only one-sixth and one-fourth respectively of the total isotope was present in the carboxyl adjacent to the keto group; it should have contained half of the total, if a symmetrical tricarboxylic acid had been the precursor. Likewise, in glutamic acid isolated from the proteins of animals which had received carboxyl labeled acetic acid the heavy carbon was not evenly distributed between the two carboxyl groups (183). The C<sup>13</sup> content of the carboxyl adjacent to the amino-group accounted for only one-fourth of the total, whereas it should have contained half of the total if the  $\alpha$ -ketoglutarate arose from a symmetrical precursor. Thus, in the intact animal also, the  $\alpha$ -ketoglutarate formed in the process of acetate oxidation is derived from an intermediate in which the orientation of the labeled carbon is preserved. The much higher isotope content of glutamic than of aspartic acid isolated from this experiment suggests that only a small fraction of the isotope incorporated into the glutamic acid was contributed by CO<sub>2</sub> assimila-

tion. The carbon chain of aspartic acid is believed to be furnished by oxalacetate. If isotope had entered exclusively in the form of  $\text{CO}_2$  by the carboxylation of pyruvate to oxalacetate, then aspartate should contain not less but as much or more heavy carbon than the  $\alpha$ -ketoglutaric acid which is subsequently formed from oxalacetate by way of a tricarboxylic acid.

The results obtained with labeled substances eliminate a symmetrical molecule as the initial condensation product, but do not indicate which tricarboxylic acid is involved. In addition to isocitrate and cis-aconitate, the two acids which according to present views are the most likely intermediates, oxalosuccinate has become a possible intermediate since Ochoa (168) has demonstrated reversibility of the isocitrate-oxalosuccinate conversion. Citric acid has a symmetrical carbon chain but the possibility still exists that a citric acid which is substituted at one of the two primary carboxyl groups lies on the main path of the cycle. The suggestion of Wieland and Rosenthal (176) that the citric acid formed in kidney from acetoacetate might have arisen secondarily from a proicitric acid containing eight carbon atoms has been mentioned. Lynen (188) finds the formation of citrate from acetate and oxalacetate by yeast to be promoted by the simultaneous oxidation of succinaldehyde and believes that the function of this reaction is to convert either one of the components into a more reactive derivative. If the condensation takes place with an acetyl compound  $\text{CH}_3\text{COR}$ , a substituted unsymmetrical citrate molecule may result, which could be converted to cis-aconitate without elimination of the substituent R. Lynen has pointed out that the data of Sonderhoff and Thomas (127) on the formation of labeled citrate and succinate in yeast, also rule out a symmetrical citric acid. Succinate formed from trideuterioacetate by way of a symmetrical tricarboxylic acid could not have contained more than one atom of deuterium, but was actually found to contain two atoms. The suggestion that an unsymmetrically substituted citric acid derivative is initially formed would also account for the unequal distribution of isotopic carbon in  $\alpha$ -ketoglutarate formed from labeled acetate or acetoacetate. The question concerning the identity of the initial reaction product might be clarified materially if it were possible to establish whether citrate formation occurs only in systems which contain the enzyme aconitase. According to Krebs (162) this enzyme is absent from yeast. In this case the citric acid arising in the course of acetate oxidation by this organism could not have been formed secondarily from isocitrate or cis-aconitate but must have been the primary condensation product.

A discussion of the rôle of the citric acid cycle in carbohydrate metabolism is beyond the scope of this review, but it may be pointed out that no evidence exists to support the contention that the tricarboxylic acid formed in the oxidative metabolism of pyruvate is identical with that arising from fatty acid oxidation. The finding of Martius (189) that oxalocitramalate, the condensation product of pyruvate and oxalacetate, is not attacked by kidney tissue is hardly sufficient evidence to rule out the occurrence of intermediate  $C_7$  tricarboxylic acids. As the experience with yeast has shown (190, 157), the lack of metabolic activity of a substance added to a biological system may be apparent only and

may be caused by inability of the substance to penetrate cell walls. This is particularly true for polycarboxylic acids. Furthermore, there are other C<sub>7</sub> acids which could conceivably arise from pyruvate and oxaloacetate and which have not been tested biologically (formylcitric, oxalocitraconic acids).

The conclusion that the tricarboxylic acid cycle provides an important mechanism for the complete oxidation of fatty acids in the intact animal is based on evidence of indirect nature. The appearance of isotopic carbon in the glycogen of animals fed labeled fatty acids and in the dicarboxylic amino acids after feeding of acetate is not by itself indicative of the mechanism responsible, but is most reasonably ascribed to pathways which lead to the intermediary formation of tricarboxylic acids. It is not possible at present to determine what portion of the fatty acids is oxidized in the intact animal by this route. From the high isotope level in glycogen in the experiments of Buchanan et al. (120) and of Lorber et al. (121) it is clear however that a significant fraction of acetate and butyrate respectively was metabolized by way of the tricarboxylic acid cycle. In these experiments with starved animals, ordinary glucose was given simultaneously with the labeled fatty acids in order to insure glycogen deposition. The labeled glycogen arising from the labeled fatty acids was therefore diluted by glycogen formed directly from dietary glucose.

The finding of Wood et al. (182) that the same isotope distribution in glucose obtains with butyrate labeled at carbon atoms 1 and 3 as with carboxyl labeled acetate demonstrates the breakdown of butyrate to two acetyl groups and furnishes additional evidence for the view that fatty acids are degraded by successive removal of two carbon units. These data are not in agreement with the suggestion of Blixenkrone-Moeller (191) that butyrate might be oxidized to succinate directly by  $\omega$  oxidation.

The results obtained during the past few years have not only provided the basis for a reasonable scheme of the total oxidation of fatty acids but have also clarified the controversial relationship of fat and carbohydrate metabolism. From the findings discussed above it may be concluded that fatty acids are convertible to carbohydrate in a restricted sense. The split products of fatty acid oxidation contribute to carbohydrate synthesis but the fatty acids by themselves are incapable of causing an increased formation of glucose or glycogen because the mechanism by which the fatty acid carbons find their way into glucose requires the participation of carbohydrate itself. This explains why in the starved or diabetic animal fatty acids and acetate do not show a glycogenic effect.

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