RADIOISOTOPE STUDIES OF FATTY ACID METABOLISM

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PREFATORY CHAPTER

INCREASES in our knowledge of fatty acid metabolism can be divided into three periods much as is that of fatty acid chemistry[1]. Disregarding the earliest inklings of knowledge gained by animal breeders, the first period might be said to extend from the first experimental proof (in 1860) that fat may be synthesized from carbohydrate[2] up to the time of the first world war. During this period advances in knowledge were made possible by the easy recognition and identification of lipid material by solubility, staining and other properties. Gross changes were readily followed by such techniques. However, during the interwar years, fatty acid metabolism was largely neglected. Separation and identification of individual acids, even when large amounts of material were available, could be accomplished only roughly and with great difficulty. Maintaining fatty acids in aqueous solutions at pH values compatible with living cells made *in vitro* studies difficult. Indeed, the actual forms in which fatty acid might be presented to the cell was a matter of conjecture. Consequently this field of research lagged, while knowledge of carbohydrate and protein metabolism made great gains.

During and after the second world war, interest in fatty acids revived. Advanced analytical and separation techniques readily permitted separations of lipids and their component fatty acids on very small samples. New methods of isolating, purifying and handling enzyme preparations enabled many complex reactions to be carried out *in vitro* by the use of a series of fairly pure enzymes and cofactors, much as the organic chemist uses pure chemicals in synthetic ventures. Of particular importance was the availability of a variety of isotopic tracer elements, stable and radioactive, which, for the first time, permitted the following of molecules and atoms through the complex pathways of various metabolic processes.

For the research worker in the lipid field, the most important of these tracer elements is the long-lived radioactive carbon isotope, C^{14}. Prior to its availability, the work of Schoenheimer with deuterium[3] and of Hevesy and Hahn[4] with radioactive phosphorus, P^{32}, demonstrated

that the fats of the body are not the inert storage substances they were originally presumed to be, but are actually in a state of fairly rapid fatty acid exchange. Since, in this state, there may be no net change in the amount of lipid, it is obvious that tracer techniques are required to reveal many of the alterations which are now known to occur continuously in the mass of lipid deposits. Neither deuterium nor phosphorus is an ideal tracer for fatty acids, the former being at times too labile and the usefulness of the latter being restricted to studies of phospholipid metabolism. C^{14} is, however, quite suitable and, moreover, carbon-labeled substances have been employed in most tracer studies on fatty acids.

The purpose of this monograph is to show how tracer studies have contributed to our knowledge of fatty acid metabolism. Where most of the pertinent information has been obtained by such methods, a fairly complete picture is presented. However, no attempt is made to more than outline ancillary information obtained by other techniques. Moreover, no attempt has been made to present a complete coverage of the literature, but only of those papers which the authors considered pertinent to the subject under discussion. In spite of the hazards imposed by the rapidly expanding scientific literature, it is hoped that the finished volume contains a fairly comprehensive discussion of recent advances in our understanding of the complex ramifications of fatty acid metabolism. The great importance of isotope-labeling techniques to progress will soon be obvious. Although it would perhaps be imprudent to suggest that many of the findings discussed here could not have been revealed without the availability of suitable isotopic tracer elements, there can be little doubt that the problems involved would otherwise have been much more difficult to solve and hence that their solution would have been regrettably delayed.

It seems likely that at the present writing (July, 1958) investigations in this field have only begun and that in ten years this book will serve simply as an account of pioneering ventures in what soon proved to be an exceptionally fertile area of inquiry.

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2. LAWES, G. B. and J. H. GILBERT, *J. Roy. Agric. Soc. Eng.* **21**, 433 (1860).
3. SCHOENHEIMER, R., *The Dynamic State of Body Constituents*, Harvard Press, 1942.
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CONTENTS

CHAPTER I

DIGESTION AND ABSORPTION

THE impact of the use of radioactive tracers in metabolic studies was not felt in the lipid absorption field until about 1950, when it became apparent to research workers in several laboratories that the vexing problems remaining in this field might be solved by the use of these techniques. It had also become painfully evident that few important advances had been made in the previous half-century and that the same arguments which had been pursued so eloquently by Munk and Pfluger in 1900 [43, 45] were merely being carried on by their heirs. Nevertheless, one series of reports had appeared which led directly to the decisive experiments of the past ten years. These were the papers of Frazer proposing and defending the "partition" theory of fat absorption [30, 31]. According to this theory, fats were only partially hydrolyzed in the small intestine. The highly emulsified mixture of fats, partial glycerides and fatty acids thus formed was absorbed into the lacteals and passed into the systemic blood through the thoracic duct. The bulk of the fatty acids split off in this process were supposed to be largely absorbed into the portal system.

This theory was contrary to conceptions widely espoused at the time of its proposal and consequently brought about a flurry of experiments primarily designed to disprove it. The early experiments with radioactive carbon probably belong in this category.

EARLY EXPERIMENTS

Following the pioneering work of Hevesy and his co-workers [37], the earliest experiments with radioactive tracers in lipid metabolism were conducted with P^{32} and were consequently limited to studies of the phospholipids and their precursors and derivatives. However, since little or no information was available at the time from experiments with radioactive carbon in the various types of lipids, interpretation of these experiments was difficult and will be considered later in the light of subsequent findings.

That radioactive carbon might aid in solving the problems of fat

absorption was demonstrated by Bergstrom, Borgstrom, Carlsten and Rottenberg[4] who, in a preliminary communication, reported experiments in which cats were fed stearic-1-C^{14} acid diluted with inactive corn oil (or with inactive corn oil hydrolysate) or incorporated into corn oil triglycerides. In the experiments in which free fatty acids were fed, the triglycerides isolated from the lymph of the animals were as active as in those in which the labeled triglycerides were fed. It was thus evident that, at least in the case of stearic acid, fatty acids were absorbed into the lymphatic system regardless of the form in which they were fed. However, poor recovery of activity and certain unexplainable differences in phospholipid activities did not permit more detailed conclusions to be drawn.

SITE OF ABSORPTION

The problem of fatty acid transport in the lymph was soon solved by a combination of tracer techniques with those involving cannulation of the thoracic and intestinal lymph ducts[14], which permit the collection of lymph from unanaesthetized rats.

Studies were undertaken in Chaikoff's laboratories in which C^{14}-labeled fatty acids of various chain length were fed to rats either as triglycerides[12, 23] or as fatty acids dissolved in corn oil[12, 23, 11]. In whatever form fed, the longer chain fatty acids appeared almost exclusively in the lymph, while those with less than 12 carbon atoms were transported to an increasingly smaller extent in this manner. Experiments involving pentadecanoic-5-C^{14} acid[23] demonstrated that the odd-chain fatty acids are not exceptional in this respect. A summary of these results is shown in Table 1. The obvious question raised by these experiments was the fate of the large proportion of the shorter chain acids not appearing in the lymph. This was at least partially answered by experiments in which the amount of a fed labeled fatty acid found in plasma from the portal vein was compared with that extracted from plasma taken simultaneously from the inferior vena cava[39]. For decanoic acid the ratio varied from 1·3 to 9·7, while for palmitic acid, it averaged 1·0 (0·9–1·2). It is evident from these experiments that the long-chain fatty acids are absorbed via the lymphatics regardless of the form fed and that the short-chain acids are largely absorbed directly into the portal blood. The transition point seems to be in the fatty acids with chain-lengths of 8 to 12 carbons and corresponds to those fatty acids for which the partition coefficients between water and fat approach

unity. Thus Frazer's partition theory was confirmed in a modified form, but not in the original sense that all fatty acids are absorbed into the portal system.

TABLE 1. PER CENT OF FATTY ACIDS OF VARIOUS CHAIN LENGTHS APPEARING IN THE LYMPH [11, 12, 23]

Chain length	Form administered	Per cent absorbed	Per cent in thoracic duct
18	Acid	36–54	84–90
16	Triglyceride	81–95	70–92
15	Acid	78–90	84–93
14	Acid	80–91	59–82
12	Acid	Complete	15–55
10	Acid	Complete	7–19

DIGESTION

Since all long-chain fatty acids, in whatever form fed, were thus shown to be largely absorbed via the lacteals and to appear in the intestinal or thoracic lymph, it became possible to investigate the form in which fats were absorbed from the intestine by examining the transformations which occurred in them during passage from the intestinal lumen to the lymph. This was, of course, the crux of the argument which had been carried on for so long.

In a continuation of their previous experiments, Borgstrom [18, 19] and Bergstrom, Borgstrom and Rottenberg [6] conducted feeding experiments with rats, using carboxy-labeled palmitic and stearic acids either free or esterified. Again it was found that regardless of the form fed, the absorbed labeled acids appeared largely in the lymph and largely as triglycerides. Some differences in the amounts of lymph phospholipid formed from the two acids was noted (11 per cent from stearic and 4 per cent from palmitic), but this seemed to be a function of the nature of the fed acid rather than the form in which it was fed. In comparable studies on a patient with chyluria and patients with thoracic duct cannulae, Blomstrand and Ahrens [9] and Blomstrand, Dahlback and Linder [10] administered carboxy-labeled palmitic and oleic acids [9] and stearic and linoleic acids [10] and investigated the distribution of radioactivity in the various lymph lipids. With palmitic, oleic and linoleic acids about 90 per cent of the activity appeared in the lymph trigly-

cerides and only 4 per cent in the phospholipids. Stearic acid behaved differently in that 20 per cent of the ingested activity was found in the phospholipid fraction, confirming the rat experiments. In the case of linoleic and stearic acids, the activity of the fatty acids in the α and β positions of the lymph lecithin was measured[10]. With linoleic acid about 75 per cent of the activity was in the α position while with stearic acid about 80 per cent was found in the β position, indicating that the selectivity of the α and β positions of the lecithin molecule for unsaturated and saturated fatty acids is established in the intestinal cells. Several other important results were obtained in these studies. When labeled stearic acid was fed as the cholesterol ester, the lymph cholesterol esters had approximately the same relative activity as when the acid was fed mixed unesterified with corn oil or trans-esterified with corn oil[18]. This meant that the sterol esters were completely hydrolyzed before absorption or were completely trans-esterified during absorption. As will be seen, however, esters of monohydric alcohols are probably completely hydrolyzed before absorption, a fact which does not reveal the extent of triglyceride hydrolysis. It was noted in all experiments that when a labeled acid was fed mixed with the fatty acids from corn oil, absorption was slower than if the acid were fed in unhydrolyzed corn oil or incorporated into this vehicle by trans-esterification. This proved to be an effect of free fatty acids on gastric emptying which had been known for some time and thus did not reveal any differences in absorption mechanism. However, an interesting result was obtained when the rate of appearance of activity in the lymph fatty acids was measured following administration of carboxy-labeled stearic acid either mixed with corn oil or trans-esterified with corn oil. In the former case, the maximum activity appeared earlier than in the latter and reached greater values. Although it was not possible to interpret this result completely at that time, it seems evident, in the light of more recent findings, that since free fatty acids contribute to the emulsification of the absorbed lipid, they may be absorbed with the first fractions before further hydrolysis (and hence dilution) proceeds to an appreciable extent. Thus, when active fatty acids are fed with triglyceride, they may be quickly incorporated into the first finely emulsified and consequently absorbable particles.

The experiments of Reiser and his co-workers[46] served to resolve many of the problems which were at that time ripe for solving. A synthetic fat prepared from glycerol labeled with C^{14} and conjugated

linoleic acid was fed to rats. It had already been shown by Bernhard, Wagner and Ritzel[7] and unwittingly by Favarger, Collet and Cherbuliez[27], both using deuteroglycerol, that free glycerol is not used to any extent in the esterification of fatty acids in the small intestine. It was therefore significant when, in Reiser's experiments, 55 to 74 per cent of the fed glycerol-α-C^{14} appeared in the lymph. It was evident that this glycerol had been absorbed in combination with fatty acid. In order to determine what type of compounds had been absorbed, the experiment was repeated with the addition of the fully saturated triglyceride, bayberry tallow, to the fed mixture. The lymph glycerides obtained from these animals were separated roughly into largely saturated and largely unsaturated fractions by crystallization from alcohol-acetone mixtures. Calculation of the ratios of glycerol-C^{14} to conjugated fatty acid in these fractions confirmed the previous evidence that no completely hydrolyzed glycerol was reutilized and revealed that the lymph triglyceride glycerol had been absorbed largely as monoglyceride. Moreover, in more recent experiments, Reiser and Dieckert[47] found, using tripalmitin labeled with C^{14} in both the glycerol and fatty acid moieties, that only about 3 per cent of the triglyceride remained completely unhydrolyzed during digestion and absorption. Admittedly, these experiments were conducted on only one type of animal under one set of conditions. However, they confirmed the ideas expressed previously on not nearly so good evidence, that total hydrolysis was not a prerequisite to fat absorption.

ACTION OF PANCREATIC LIPASE

The reasons for absorption of fatty acids in the form of monoglycerides soon became apparent from the results of experiments on the action of pancreatic lipase on triglycerides *in vitro* and *in vivo*. Frazer[31] had advanced the hypothesis that the action of pancreatic lipase was incomplete because of inhibition by free fatty acids, which tend to accumulate at the oil–water interface and block further hydrolytic action. Borgstrom, however, using *in vitro* conditions, found that initial concentrations of free fatty acids ranging from 0·5 to 22 per cent had no influence on the rate of hydrolysis of triglycerides[17]. In further studies on this subject, Borgstrom and others[20, 21, 22, 27] conducted both *in vivo* and *in vitro* studies on the changes induced by pancreatic lipase on various combinations of glycerides and fatty acids.

When, under *in vitro* conditions, olive oil containing a small amount of oleic-1-C^{14} acid was subjected to the action of lipase, activity appeared in the glyceride fraction, indicating either trans-esterification or true synthesis. The activity of the fatty acids incorporated into triglycerides approached, but did not exceed 60–70 per cent of that of the free fatty acids. Similarly, for diglycerides this value was 50 per cent and for monoglycerides, 20 per cent. When the substrate was active palmitic acid trans-esterified with olive oil, the value was 100 per cent. When a synthetic triglyceride containing active decanoic acid in the 1,3-positions was treated in a similar manner, the triglyceride activity remained at 60 per cent of that of the liberated fatty acids. Similar results were obtained in *in vivo* studies. These results indicated that there were only two exchangeable fatty acid residues in triglycerides, one in diglycerides and none in monoglycerides. The hypothesis was advanced that pancreatic lipase is specific for the primary hydroxyl groups of glycerol and that the secondary or 2-position is not readily hydrolyzed by this enzyme. The 20 per cent activity found in the monoglyceride was interpreted to mean a conversion to 80 per cent 2-monoglyceride and 20 per cent 1-monoglyceride. Later results make more likely the possibility that a slow isomerization of the 2- to the 1-isomer takes place under these conditions. Thus hydrolysis of triglycerides in the small intestine may take place as follows: triglyceride $\rightarrow$ 1,2-diglyceride $\rightarrow$ 2-monoglyceride $\xrightarrow{\text{slowly}}$ 1-monoglyceride $\rightarrow$ glycerol.

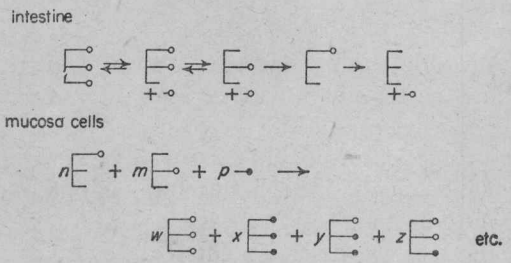
In the first three steps, the synthetic reaction also occurs, as shown by Borgstrom[22] in experiments in which fed inactive monoglyceride incorporated active free fatty acids to form active triglycerides. However, in the fourth reaction the equilibrium lies well to the right and little if any free glycerol is esterified by pancreatic lipase. These findings were confirmed *in vivo* by isolation of the 1,2-diglyceride during triglyceride digestion[21] and have since been amply supported by the work of Mattson and his co-workers[40] using different techniques.

It is of further importance that the *in vitro* action of lipase on triglyceride could thus detect whether it was randomly or specifically labeled by the ratio of the activities of the fatty acids released to those of the unhydrolyzed triglycerides. It was shown by Borgstrom in this way that lymph triglycerides formed following the feeding of active fatty acids are completely randomized. The randomization evidently took place during passage of the glycerides and fatty acids through the

intestinal wall, since pancreatic lipase was not able to carry out this reaction. Some doubt was also cast by this information on the quantitative significance of Reiser's results, since it appeared possible that some of the re-esterification of monoglyceride occurred, not in the intestinal cells but in the lumen.

Whether the glyceride leaving the lumen and passing into the epithelial cells is actually a monoglyceride or a mixture of mono- and diglycerides is thus not completely settled. However, these experiments confirmed and gave a mechanistic basis for the evidence that triglycerides are largely broken down to monoglycerides and fatty acids before absorption.

The studies outlined above have suggested strongly that fat digestion and absorption, at least in the rat, proceeds by the following mechanism:



PARTICIPATION OF PHOSPHOLIPIDS

Of course, in no case does the total absorbed fatty acid appear in the lymph as triglyceride. Depending on the particular acid involved, different proportions of the labeled acid have been found in the lymph as phospholipid. Thus, when stearic acid was fed, about 11 per cent of the active acids in the lymph were in the form of phospholipid [18]. Similar experiments by Borgstrom and his collaborators and by Blomstrand have revealed that for oleic acid, the proportion is 2 per cent [5], while for palmitic [16] and linoleic [8] acids, it is about 4 per cent. Despite this small proportion of absorbed fatty acid actually appearing in the lymph as phospholipid, it had seemed attractive to many of the early workers in the field that phospholipid might be an obligatory intermediate in fatty acid absorption and conversion to lymph triglycerides.

These ideas originated in the observation that lymph phospholipids increased markedly during active fat absorption [28, 51, 55] but could not be definitely substantiated or disproved by techniques then in use. The availability of radioactive phosphorus, therefore, provided an opportunity to perform decisive experiments.

Several early experiments, involving the administration of inorganic P^{32} (as sodium phosphate) indicated that a higher turnover rate of lipid phosphorus occurred during fat absorption than in the fasting state [1, 29]. It was, however, shown that the actual amount of phospholipid present does not increase during fat absorption. Therefore, any increase in turnover rate had to account for the passage of the total absorbed fatty acids through this form. In an experiment designed to test this possibility, Zilversmit, Chaikoff and Entenman [55] measured changes in the relative specific activity of small intestinal phospholipids

$$\frac{\text{sp. act. of phospholipid } P^{32}}{\text{sp. act. of acid sol. } P^{32}}$$

of dogs and rats during fasting and active fat absorption. In the case of the dogs, neither the amount nor the turnover rate of the phospholipid phosphorus of the mucosa or villi were affected by fat absorption. In the rat, some changes occurred, but these were too small to account for passage of all fatty acids through this form if phosphorus turnover could be shown to be an adequate measure of such turnover. That this might not be the case was quickly pointed out by other workers in the field. In the first place, it was possible to imagine that fatty acid turnover did not involve phosphorus turnover, especially in view of the findings of Hanahan and co-workers [34, 35] that the α' position in lecithins is specific for unsaturated acids, which might therefore turn over without affecting the integrity of the remainder of the molecule. Moreover, it was pointed out that the turnover rate of total phospholipid phosphorus might be the result of the turnover rates of inactive and very active compounds and that the true picture could be gained only by examining the latter. The latter argument seems specious in view of the complete lack of evidence in the case of the dog. The former argument has been largely denied by the experiments of Reiser and Dieckert [48], who found that following the feeding of tripalmitin labeled with C^{14} in both acid and glyceride portions, the relative specific activity of intestinal mucosa and lymph glyceride and phospholipid glycerol could be interpreted only to show that triglycerides were precursors of phospholipids rather than the reverse. It thus appears, in the light of present information

that the phospholipids are not precursors of triglycerides during fat absorption, but are formed simultaneously, probably as part of the lymph transport system for the newly absorbed fats.

ORIGIN AND FATE OF GLYCEROL

From the experiments of Reiser and others, it was apparent that some of the glycerol of ingested fat was absorbed as glyceride and became part of the lymph lipids. However, there was little evidence from these experiments as to the fate of glycerol freed of all fatty acids by hydrolysis in the lumen nor the origin of the extra glycerol incorporated into lipids presumably in the cells of the intestinal epithelium. One fact was clear from these experiments as well as from those of Favarger and co-workers, using deuterium-labeled glycerol[24, 25]. Free glycerol released by hydrolysis was not incorporated to any extent into the lipids appearing in the lymph during fat absorption. This is not surprising in view of the work of Gidez and Karnovsky[33] showing first that the released glycerol is absorbed and oxidized much more rapidly than the fatty acids and second that little of this glycerol is available for synthetic processes, even for synthesis of liver glycogen. That some incorporation does take place, however, has been shown by the same authors[32] and by Morehouse, Skipski and Searcy[41]. In both cases, fed or injected glycerol was incorporated into the lipids of the small intestine, although to a much smaller extent than into liver lipids[32]. With these facts in mind, Reiser and Williams[49] conducted experiments in which (palmitoxy-1-C^{14})-hydroxyacetone was fed and the active compounds isolated from lymph. Of the 25 per cent of this material absorbed, all the activity appeared in the triglyceride fraction, thus showing the conversion of hydroxyacetone esters to glycerol esters in the intestinal epithelium. However, these experiments could not be interpreted to implicate dihydroxyacetone as an obligatory intermediate in triglyceride formation nor could the previous results on glyceride glycerol be used to prove that endogenous glycerol could not be used for this purpose. As a matter of fact, it appears likely that endogenous L-α-glycerophosphate might serve as the source of glyceride glycerol.

It appears that if this problem is meaningful, it will have to be solved by other types of experiments.

DETERMINATION OF STATE OF ABSORPTION

Tracer methods have been adopted only gradually for use in studies of rate and extent of absorption. This is partially true because most of these studies are conducted on human patients or with this ultimate aim, and the use of tracers in routine human studies has usually not seemed justified. Moreover, as will be seen below, many of the tracer studies are difficult to interpret since the data obtained from them result from more than one process.

Generally speaking, information on rate and extent of fat absorption has been obtained by one of the following methods:

Fat balance studies: for human studies, this method, involving daily analysis of fecal fat for a fairly long period of time, gives the only truly accurate picture of fat absorption in man. However, it is tedious and time-consuming and might not benefit materially from the use of tracer techniques.

The Cori technique: for animal studies only, this method, involving excision of the total gastrointestinal tract followed by analysis of the remaining administered fat, is probably the most accurate in use at present. Use of C^{14}-labeled fatty acids may have increased the accuracy of the method [53].

The appearance of the administered fat in the blood: this method can be used for both human and animal studies and is well adapted to the use of tracers. However, interpretation may be difficult since the blood fat level is the result of several processes involving both absorption and removal.

The appearance of carbon from ingested fat in the respiratory carbon dioxide: this method is especially suitable for tracer techniques, but suffers from all the disadvantages of the last-mentioned method plus the additional complication of degradation rate.

The tracer method presently used most widely for human studies is that employing I^{131}-labeled fat, since the relatively short half-life (8 days) of this isotope shortens the period of exposure of the patient to radiation following the experiment. Iodination of triglyceride containing unsaturated fatty acids results in little apparent change in properties, and the absorption rate of this fed fat can be followed from the changes in radioactivity of the blood. Total absorption can be estimated from the fecal and urine counts of subjects which have received Lugol's solution (iodine-potassium iodide) to block thyroid uptake. Such studies have