LIPOGENESIS IN THE RAT LIVER SLICE

by

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CHAPTER I

INTRODUCTION

Origin of This Study

With the advent of isotopic tracers a whole new array of techniques has become available for a fresh attack on many of the perplexing problems of lipid metabolism. The use of these tracers in the intact animal has made possible detailed investigation of certain previously uncharted metabolic pathways. More recently, however, most of the attention has been focused on the more refined but less organized systems, i.e., the tissue slice, the homogenate and purified enzyme. This shift of interest from in vivo to in vitro methods was natural in light of the many inherent problems associated with studies involving the intact animal. Difficulties encountered in the intact animal studies are briefly discussed below.

First, in vivo studies, even with isotopic labeling, are subject to much interpretation since the multitude of homeostatic mechanisms, both neurovascular and endocrine, which complicate the intact organism are fully operative and not subject to precise control.

Second, in vivo experimentation often requires rather gross techniques involving large volumes and lengthy analyses. In addition, adequate control measures dictate prolonged nutritional and environmental preparation of experimental animals.

For these and other reasons this laboratory has, in recent years, been one of the few remaining sources of in vivo information on lipid metabolism. From the beginning one of the aims of our laboratory has been to correlate this in vivo information with in vitro results obtained by other investigators. Unfortunately, animal pretreatment, experimental design and analytical techniques among the various laboratories have been so varied that any satisfactory correlation between our intact animal data and that reported from in vitro studies has not been possible.

It became apparent that the only way to accurately correlate in vivo and in vitro data was to pursue both techniques in the same laboratory, using identical materials and methods in so far as was practicable. It was clear that our in vitro approach could not proceed far down the scale of tissue subdivision without sacrificing much of our goal and for this reason the more conservative tissue slice technique was adopted. The slice technique, occupying, as it does, an intermediate position between the intact animal and the crystalline enzyme preparation, was well suited for our purposes. With the tissue slice, cell boundaries remain intact and the use of appropriate media makes possible a quasi-physiological intra and extracellular environment. Thus the study of lipid metabolism is possible without the influence of variable homeostatic controls and yet cellular integrity has not been entirely sacrificed. By identical pretreatment and analytical techniques, the qualitative information obtained from the slice should be more readily correlated with quantitative data obtained from the intact animal.

In undertaking these liver slice studies, every attempt has been made to simulate our intact animal methods. This has involved careful

controlled-feeding of the rats prior to sacrifice, use of the same isotopic tracer materials and identical biochemical and radio-assays. In addition, an attempt has been made to duplicate in vivo experiments wnenever possible. It is recognized that the slice technique is more amenable to the study of certain detailed phases of lipid metabolism than is the intact animal and no attempt has been made to limit our in vitro studies to previously performed in vivo experiments.

Theory of the Slice Technique

The following discussion has been assembled from a pool of information which has resulted from four years of study of the tissue slice technique. Credit has been given whenever applicable but much of this material has grown out of our own observations and discussions and for this reason references are frequently lacking. At the present time only three critical analyses of the slice method are available,[1,2,3] including the original descriptions by Warburg, and since many of the factors outlined below are inadequately dealt with in these works, we have included our own supplementary observations.

As has already been pointed out, the tissue slice represents an attempt to free surviving cells from neurovascular and endocrine influences while maintaining intact functioning cells in as nearly a physiological extracellular environment as possible. This technique, developed in most of its details by Warburg and his co-workers in their studies of tumor metabolism,[1] represents organized surviving tissue, the metabolism of which qualitatively if not quantitatively, reflects that of the original tissue. Further, the slice method allows for controlled variations in

the suspending medium in addition to chemical analysis of both the intact slice and the suspending medium for changes in metabolite content.[4]

The preparation of metabolically active slices as well as the provision of a compatable environment are based on certain theoretical considerations, the details of which are briefly outlined below.

Tissue Slice Thickness

Since the rate of diffusion of gases and metabolites will be determined in part by the thickness of the tissue slices, it is essential that they be of uniform thickness within certain limits. These limits are determined by the diffusion constants of the reacting substances, the rate of metabolism of the tissue in question, etc. As derived by Warburg,[4] the limiting thickness in cm (d') for oxygen consumption of slices of a given tissue can be calculated from the equation:

$$d' = 8C_o \frac{D}{A}$$

where D = diffusion constant for oxygen in ml (NTP = 1.4×10^{-5} ml/min at 38°C through tissue of one square cm cross section.

A = rate of respiration $\frac{\text{ml } O_2 \text{ uptake}}{\text{ml tissue x min.}}$

C_o = oxygen concentration outside the slice (in atmospheres).

Taking 5×10^{-2} as A for liver slices, 1.4×10^{-5} as D and 1.0 as C_o for pure oxygen, d' is calculated to be 4.7×10^{-2} cm or 0.5 mm as the limiting thickness for liver slices in pure oxygen. Under these conditions, the oxygen tension at the center of the slice will be about 0.6 atmospheres.[4]

Temperature of Tissue and Medium

In order to reduce the oxygen requirements of the tissue while it is being sliced, it is important to chill both the uncut organ and the slices as they are cut. This serves two purposes. First, the devascularized organ is far too thick to allow diffusion of oxygen further than the outermost layers of cells. The presence of capsular tissue surrounding most organs precludes even this small contribution. As a result, the vast majority of cells within the organ are immediately subject to a condition of relative anoxia which, in the presence of any appreciable metabolic activity, would quickly lead to cellular disturbances making the tissue unsuitable for use. This avascularity can be at least partially compensated by chilling the uncut organ and reducing metabolic activity to a level where hypoxia will be less significant.

Second, once the slice is cut, oxygen diffusion improves and becomes more adequate for metabolic requirements and the utilization of available substrates or energy stores begins. Since it is desirable to preserve as much reserve energy as possible until the slice can be suspended in the test medium in the Warburg flask, the cut slices must be kept chilled until the actual incubation period is begun.

This chilling of the organ and cut slices can be readily accomplished by storing them on cracked ice until transfer to the Warburg flask can be completed. Details of a convenient chilling procedure can be found in Chapter II.

The temperature of the incubation bath should be as nearly physiological as possible but considerable variation will be found when specific functions of a cell are being studied. Certain reactions may

occur most rapidly and with greatest yield at temperatures several degrees above or below the mammalian body temperature of 37°C. Most studies are however conducted at temperatures ranging between 37° and 38.5°C.

A more important consideration is the uniformity of temperature within the bath since, as will be detailed later, a 0.5°C variation in temperature between two flasks in the bath will result in a significant error in oxygen utilization measurements due to the resulting change in flask constants. It is usually considered essential that all parts of the bath be maintained within ± 0.05°C limits.

Suspending Medium

The commonly employed Krebs-Ringer solution[5] is so constituted as to closely approximate the ionic composition of mammalian serum. This medium provides a physiological extracellular environment and insures the metabolic integrity of the surviving cells.[4]

The use of Krebs-Ringer-Bicarbonate would be most desirable since the CO_2-bicarbonate system of the extracellular fluid is the chief buffer system in the body and the absence of CO_2 limits the maximum respiratory activity of tissue slices.[2] In most applications however, Krebs-Ringer-Phosphate is preferable since the presence of bicarbonate in the medium complicates manometric measurements of oxygen utilization and makes more difficult the quantitative estimation of CO_2 production.

Krebs-Ringer-Phosphate medium is made from aliquots of the following solutions:

1). 0.154 M (0.90%) NaCl

2). 0.154 M (1.15%) KCl

3). 0.11 M (1.22%) $CaCl_2$

4). 0.154 M (2.11%) KH_2PO_4

5). 0.154 M (3.82%) $MgSO_4 \cdot 7H_2O$

6). 0.1 M Phosphate buffer (17.8 gm. $Na_2HPO_4 \cdot 2H_2O$ + 20 ml 1 N HCl, diluted to 1 L).

To prepare a working Krebs-Ringer solution, the following amounts of the above standard solutions are mixed:

100 parts of solution 1).
4 parts of solution 2).
3 parts of solution 3).
1 part of solution 4).
1 part of solution 5).
12 parts of solution 6).

A solution prepared from the first five components, without the addition of the 0.1 M phosphate buffer, is stable in the cold about one week.[4]

In practice, the original solutions are prepared in five times the indicated concentrations (4.5% NaCl, 5.75% KCl, 6.10% $CaCl_2$, 10.55% KH_2PO_4, 19.10% $MgSO_4 \cdot 7H_2O$) which, when stored individually in the refrigerator are stable for months.[4] A stock Krebs-Ringer solution may then be prepared from these concentrated solutions in the proportions listed above (100 parts solution #1, etc.) but excluding the 0.1 M phosphate buffer. The five-fold concentration must be taken into account in preparing this stock solution (436 parts of H_2O for each 109 parts of the concentrated stock solution).

The 0.1 M phosphate buffer is freshly prepared at the time the medium is to be used and 12 parts of this buffer are mixed with 109 parts of the stock solution (an alternative method is to dilute 10 ml of the phosphate buffer to 100 ml with the stock solution). The phosphate buffered Krebs-Ringer solution is then adjusted to pH 7.4 with 0.1 N HCl or 0.1 N NaOH as required, chilled and gased with 100% O_2 for ten minutes. This gased solution should then be used within six to eight hours since bacterial growth is extremely rapid even when stored in the refrigerator.

Agitation

It has been previously pointed out that 0.5 mm is the limiting thickness for liver slices. This calculation assumes an oxygen tension of one atmosphere at the surface of the slice. It would be impossible for simple diffusion of oxygen through a non-agitated suspending medium to account for this tension but if the flask and its contents are agitated, the slice will be constantly exposed to a new surface of buffer which can provide the required oxygen tension. Dixon and Elliott investigated this requirement and concluded that a rate of 100, 2 cm oscillations of the flask per minute is sufficient for tissues requiring 600-700 uL of oxygen per gram of tissue per hour and that a rate of 138 oscillations per minute can supply adequate oxygenation to tissues requiring 1500 uL/gm./hour.[6]

Calculation of Oxygen Consumption

The conversion of manometer readings to oxygen utilization is simple in practice but is based on a rather complex calculation of the flask constant (k), a thorough discussion of which is beyond the scope of this paper. Certain general principles, however, do deserve mention.

Constant volume respirometry is based on the principle that if the temperature and volume of a reaction vessel are kept constant, the utilization or liberation of a gas by the contained reacting mixture will be reflected in an altered pressure within the enclosed system. This change in pressure can be measured by means of a manometer attached to the reaction vessel. Therefore, if one knows the gas volume of the reaction vessel, the volume of fluid in the vessel, the temperature of operation, the gas being exchanged and the density of the fluid in the manometer, it is possible to calculate the amount of gas used up (or given off), providing only one gas is being changed.[1] This consists of so calibrating the system that from the observed pressure changes one can calculate the amount of gas utilized by the formula:

$$x = h \cdot k$$

where x = volume of gas utilized in uL at $0^{\circ}C$ and 760 mm pressure

h = observed change in mm in the open side of the manometer when the closed side of the manometer is adjusted to the "zero point" (usually 150 mm or 250 mm), thus providing a constant volume within the reacting system.

k = flask constant

The flask constant (k) is calculated from the formula:

$$k = \frac{V_g \frac{273}{T} + V_f \alpha}{P_o}$$

where V_g = volume in uL of the gas phase in the flask including connecting tubes down to the "zero point" on the closed side of the manometer.

V_f = volume in uL of fluid in the vessel.

T = temperature of the bath in absolute degrees (273 + temperature in °C).

α = solubility in the liquid in the vessel of the gas involved (expressed as ml of gas/ml of liquid when the gas is at a pressure of one atmosphere at the temperature T).

P_o = 760 mm Hg expressed in terms of the manometer fluid

$$760 \times \frac{13.60}{\text{specific gravity of manometer fluid}}$$

A sample calculation follows: our flask #1 has a volume of 162.461 ml or 162,461 uL up to the "zero point" on manometer #1 (150 mm mark). To this flask we add 25 ml of suspending medium (Krebs-Ringer-Phosphate buffer), 1 ml of labeled acetate and 0.2 ml of 8.7 M NaOH, giving a total fluid volume (V_f) of 26.2 ml or 26,200 uL. The incubation temperature is 37°C and the manometer is filled with Brodie's solution.* The flask constant (k) would then be:

*23 gm. NaCl + 5 gm. sodium choleate (Merck) in 500 ml of water, density 1.033. Color with 200 mg/liter Evan Blue or acid fuchsin.

V_f = 26,200 uL

V_g = 162,461 - 26,200 = 136,261 uL

α = 0.0239 (solubility of oxygen in NaCl at 37°C)

$P_o = 760 \times \frac{13.60}{1.033} = 10,000$

$$k_{O_2} = \frac{V_g \frac{273}{T} + V_f \alpha}{P_o}$$

$$= \frac{(136,261) \frac{273}{310} + (26,000)(0.0239)}{10,000}$$

k_{O_2} = 12.05 uL/mm change in manometer

Therefore, if a difference in 10 mm is observed on the manometer, the oxygen utilization would be:

$x = hk$

$= (10)(12.05)$

$x = 120.5$ uL oxygen (0°C, 760 mm)

In the development of the flask constant (k), a value P is employed which is assumed to remain constant throughout the incubation period. This value P represented the initial atmospheric pressure. The pressure in the room and the temperature of the bath are likely to change, however, and these changes are corrected for by a thermobarometer. This thermobarometer consists merely of a flask containing water attached to a manometer; the volume of the water is not critical. Any changes in the manometer readings of the thermobarometer will then represent changes in temperature of the bath or atmospheric pressure. Correction of manometer readings from flasks containing tissue by adding or subtracting thermobarometer differences will then give the true manometer differences

for the respiring flasks which would have been observed if the bath temperature and atmospheric pressure had remained constant.

The only error involved in this thermobarometer correction is the use of the wrong flask constant when the temperature of the bath changes. If the whole bath is at a uniform but lower or higher temperature than that used to calculate the flask constants, this error is not significant. Thus, if the whole bath has dropped to 36°C from the initial 37°C, our flask constant would be 12.09 instead of 12.05 or an error of 0.33%. A more serious problem arises however if the temperature of the bath is not uniform. In this case, a flask at a temperature 1°C below another flask would indicate a pressure corresponding to about 33 uL of gas less per 10 ml of gas volume in the flask, due to the decrease in pressure exerted by the gas at a lower temperature. For this reason, the temperature within the bath must be uniform within 0.05°C (a difference of 0.05°C = 1.7 uL per 10 ml of gas volume or about 23 uL in our flask which represents about 1.5% of the hourly oxygen utilization per gram of liver slices). This requirement for uniformity of temperature within the bath necessitates vigorous stirring of the water in the bath.[4]

Techniques of Other Investigators

Current tissue slice techniques as reported in the literature display a certain uniformity despite the large number of laboratories using the procedure. Principal variations are found in the size of the reaction vessel, the method of slicing and the concentration of tracer used. A review of the techniques reported by two of the major investigators in the field of lipogenesis will serve to indicate the two principal techniques now in use.

I. L. Chaikoff and his coworkers use the following technique.[7] The rat is sacrificed by cervical fracture and the liver excised and sliced free-hand with a razor blade. The slices are collected in a petri dish containing cold Ringer-Bicarbonate solution prepared according to Krebs and Henseleit. Approximately 300-500 mg. of slices are gently blotted on a moist filter paper, weighed on a torsion balance, and placed in a 15 ml incubation flask containing 4.5 ml of the bicarbonate buffer and 0.5 ml of a solution containing 10 uM of labeled acetate per ml. Incubation is carried out for three hours at 38°C in a gas phase of 95% oxygen and 5% CO_2. The reaction is stopped at the end of the incubation period by adding 0.25 ml of 5 N H_2SO_4 from the side arm. CO_2 is collected in the center well on a roll of No. 50 Whatman filter paper moistened with 1.25 ml of 30% KOH which is injected into the center well at the end of the incubation period through a rubber policeman attached to a tube passing through the bottom of the flask and into the center well.

The following technique of Medes and Weinhouse was obtained by personal communication with Dr. Grace Medes and is, essentially, the technique employed in this laboratory.

The rat is killed by decapitation and the liver quickly removed and sliced with the Stadie-Riggs microtome. The slices are transferred to a tared crystallizing dish containing ten ml of cold Krebs-Ringer-Phosphate buffer solution. The tared dish plus slices is then reweighed to obtain the tissue weight (usually 5 gm.) and the slices plus buffer are transferred to a 250 ml reaction flask with the aid of an additional 40 ml portion of buffer. The flask is then gased with a high flow of pure oxygen for a few seconds and then attached to the manometer and

incubated for three hours with 40 uM of labeled acetate. At the end of the incubation period the flasks are acidified with 0.5 ml of 18 N H_2SO_4. CO_2 is collected on a roll of KOH-moistened filter paper in a glass tube placed inside the center well of the flask.

Review of Lipogenesis

Although a complete review of the field of lipogenesis is beyond the scope of this thesis, certain essential features will be discussed as a background for interpretation of the experimental data to be presented.

Acetate and Lipogenesis

The choice of labeled acetate as a tracer substance for the experiments to be reported in this thesis is based on the fact that this two-carbon fatty acid molecule serves as the principle precursor for the biosynthesis of both long-chain fatty acids and the complex steroid molecule, cholesterol (cf.). The fate of trace amounts of isotopically labeled acetate may therefore be interpreted as indicating the flux of these two-carbon building blocks within living tissues. The precise quantitative interpretation of some of the data so obtained is difficult however and erroneous conclusions of reaction rates may be made.

Despite this problem of interpretation, the tracing of lipogenic pathways with labeled acetate has become an acceptible as well as extremely fruitful technique, and has been responsible for much of our present knowledge of lipogenesis.

A more detailed treatment of the role of this two-carbon unit in lipid metabolism as it specifically relates to fatty acid and cholesterol metabolism will be presented below.

Fatty Acid Metabolism

Although the primary concern in this thesis is with fatty acid synthesis, knowledge of fatty acid oxidation has provided much of the foundation for more recent developments in the understanding of biosynthesis and therefore will be dealt with first.

Oxidation--In 1904 Knoop[8] tagged the hydrocarbon ends of both even-and odd-carbon fatty acids with the phenyl group and fed these phenyl-substituted fatty acids to dogs. Examination of the urine showed that when even-carbon fatty acids (C_2, C_4, etc.) were used, the end product of their oxidation was phenyl-aceturic acid, while odd-carbon fatty acids (C_3, C_5, etc.) caused the excretion of hippuric acid. Knoop concluded from this that the oxidation of fatty acids takes place at the carbon atom in the β-position to the carboxyl group.

These observations were extended and confirmed in 1909 by Dakin[9] who concluded that fatty acids in general are oxidized at the β-carbon with the splitting off of the two terminal carbons, leaving the fatty acid chain shorter by two carbons than the original acid. By successive repetition of this process Dakin considered that each molecule of fatty acid oxidized would give a four-carbon acid, butyric, which would in turn be oxidized at the β-carbon to form only one molecule of acetoacetic or of β-hydroxybutyric acid (later proven incorrect, cf.).

Knoop,[8] in his theory of β-oxidation, postulated a four-stage cycle in which the fatty acid was 1) dehydrogenated to the 2:3 unsaturated derivative which was then 2) hydrated to yield the β-hydroxy derivative which in turn was 3) dehydrogenated forming the β-keto derivative. Finally, this β-keto derivative was split at the β-position, leaving

the fatty acid chain shorter by two carbon atoms and resulting in the formation of CO_2 and H_2O.

This theory implied that the oxidation of a long-chain fatty acid must involve the formation of a whole series of shorter even-carbon fatty acids in addition to the unsaturated, β-hydroxy and β-keto derivatives of each. It seemed reasonable to expect that some of these derivatives would be present in detectable amounts. These intermediates have, however, never been isolated and sensitive radio-isotope methods have even failed to reveal traces of them.[10] The most complex intermediate found has been acetoacetic acid (C_4) which Knoop[8] explained as being the unoxidizable stump of the fatty acid chain.

As an alternative theory to explain the absence of intermediate-chain fatty acids, Hurtley[11] suggested in 1915 that oxidation takes place simultaneously at alternate carbon atoms, followed by fragmentation of the whole chain. This theory also would explain the finding of more than one molecule of acetoacetate from the oxidation of a single fatty acid molecule,[12] a finding incompatable with Knoop's original theory where acetoacetate was considered the unoxidizable stump of the fatty acid chain. Hurtley's multiple-alternate-β-oxidation theory was however never accepted since it required the insertion, in the fatty acid molecule, of a series of conjugated double bonds which would be readily detected spectroscopically. The lack of spectroscopic evidence for this conjugated system, in addition to the observation that certain fatty acids with conjugated double bonds are dietary essentials and therefore not biosynthesized (linoleic, linolenic, and arachidonic), cast much doubt on this theory.

Recently support for Knoop's sequential β-oxidation theory has been provided by experiments with fluoracetic acid. The use of fluoracetate as a blocking agent in the tricarboxylic acid cycle had been shown to be due to the condensation of the fluoracetate with oxalacetate to form fluorocitrate which was not metabolized by the enzyme systems of the cycle.[13] Fluoracetic acid was therefore highly toxic when fed to rats. In 1954, Dominquez, et. al.[14] prepared a series of ω-fluoro-fatty acids and found that only the even-carbon derivatives were toxic, indicating that the end product of the odd-carbon compounds was fluoro-propionic acid which would not need to be metabolized in the cycle and therefore was non-toxic.

Despite the accumulated body of data supporting Knoop's theory, it could not be accepted as proven until the requisite enzymes had been demonstrated. The first step in this development was made in 1948 with the report of Grafflin and Green[15] on their "cyclophorase" preparation. This cell-free preparation, consisting of nuclei and mitochondria, contained all the enzymes of both the Krebs cycle and the cytochrome system and would oxidize both short-chain fatty acids (except formic and propionic) and "Knoop intermediates" ($\alpha:\beta$ unsaturated, β-hydroxy and β-keto derivatives). Their system required "sparking" by trace amounts of one of the Krebs' cycle intermediates (α-ketoglutarate or succinate) and thus gave support to a long-established relationship between fat and carbohydrate metabolism. In addition to the previously postulated relationship of the oxidation of fatty acid-derived acetate to CO_2 in this normal pathway of carbohydrate metabolism, it appeared that the cycle was also necessary in order to activate the fatty acid oxidation process.[10]

The discovery of Coenzyme A by Lipmann[16] in 1945 and the subsequent isolation of acetyl-Coenzyme A by Lynen and Coworkers[17] in 1951 brought to a close nearly half a century of searching for this "active acetate". The —S—CO— link is a high energy bond comparable with high energy phosphate linkages and capable of performing all of the functions which had been assigned to "active-acetate."[18] One of the unique features of acetyl-CoA is that the methyl group of the acetate is rendered active making the compound extremely valuable in the biosynthesis of carbon to carbon bonds.[10]

The final links in the proof of Knoop's theory of β-oxidation of fatty acid have come within the last five years with the isolation, chiefly by Green and Lynen, of a series of enzymes capable of completely oxidizing a long-chain fatty acid. Two excellent summaries of this work are the recent papers by Crane,[19] et al. (1956) and King[10] (1956). The following outline of this enzymatic sequence is taken from the review by King.[10] Briefly, the oxidation of a fatty acid chain takes place in the following stages:

1). Activation of the fatty acid by conversion to its CoA derivative. This step is dependent on ATP for the creation of the high energy bond. The exact nature of the enzyme involved has not been determined, although an "activating enzyme" capable of coupling fatty acids to CoA has been demonstrated by Mahler, et al.[20] The energy requirement for this step may be provided by either ATP per se, or as demonstrated by Stern, et al.,[21] an activated Krebs cycle intermediate (succinyl-CoA)may exchange its CoA with the fatty acid, giving the acyl-CoA fatty acid plus de-activated succinate. This would explain the "sparking" action of Krebs cycle intermediates in initiating fatty acid oxidation as found by Green[15] in

his "cyclophorase" preparation. Since the cycle is autocatalytic once this initial energy requirement is met, only trace amounts of the Krebs intermediate are required.

2). Dehydrogenation of the acyl-CoA derivative resulting in the α:β unsaturated acyl-CoA derivative. Four flavoproteins have been shown to be involved in this step. Three of these are metallo-flavoproteins which represent the three dehydrogenases which have been isolated by Green, et al.[19] These dehydrogenases each have a different substrate specificity and their use is determined by the chain-length of the fatty acid being dehydrogenated (Enzyme G—C_3 to C_8, Enzyme Y—C_4 to C_{16}, or Enzyme Y'—C_6 to C_{16}). The fourth flavoprotein has been named ETF (electron transferring factor) and is necessitated by the fact that the above dehydrogenases cannot transfer their electrons directly to the cytochrome system but require the assistance of this intermediary transferring factor.

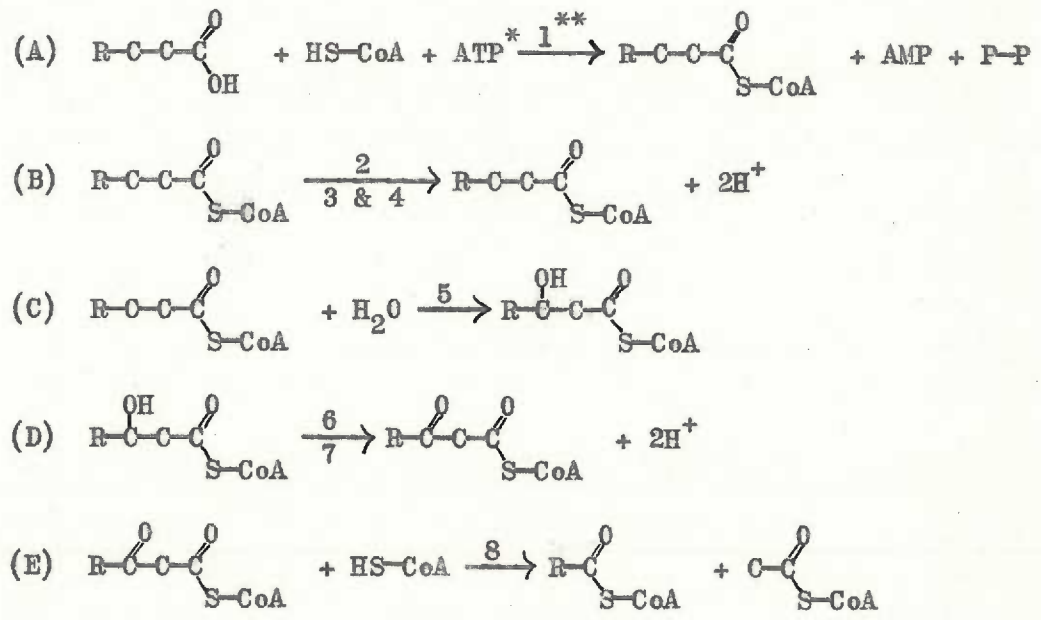
3). Hydration of the unsaturated acyl-CoA derivative to give the β-hydroxyacyl-CoA derivative. An enzyme involved in this step is crotonase which has been isolated as a crystalline enzyme by Stern, et al.[21]

4). Dehydrogenation of the β-hydroxyacyl derivative to give the β-ketoacyl CoA derivative. This step is carried out by the DPN-linked enzyme β-hydroxyacyl dehydrogenase, which has been characterized by Wakil.[22]

5). Thiolysis with cleavage of the two carbon unit by the enzyme β-ketoacyl thiolase. Less is known about this enzyme which has been demonstrated by Green,[23] and Lynen.[24] Lynen[24] has suggested that this enzyme is attached to its substrate through an —SH group and that this

linkage may persist after thiolysis has taken place, resulting in a pool of acetate-enzyme complexes which may then react with the "acetyl-CoA pool" with the reformation of acetoacetyl-CoA and the liberation of the thiolase enzyme. (This concept has particular application in the field of ketone body formation where it helps explain much of the isotope-labelling data.[10])

Diagrammatically, these steps in the fatty acid cycle may be represented as follows:



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*Succinyl CoA may replace both the HS—CoA and ATP in this step.

**1 = "activating enzyme"

2 = one of the 3 flavoprotein acyl dehydrogenases (G, Y or Y')

3 = "Electron Transferring Factor" or ETF (also flavoprotein)

4 = cytochrome system

5 = crotonase (enoylhydrase)

6 = β-hydroxyacyl dehydrogenase

7 = DPN

8 = β-ketoacyl thiolase

With the demonstration of enzyme systems capable of carrying out the sequential β-oxidation of fatty acids, the theory of Knoop is now generally accepted. The failure to demonstrate "Knoop intermediates" is explained by their presence in very small amounts as the CoA derivatives and the failure to find short-chain fatty acids has been explained by Green[25] on the basis of the substrate affinities of the enzymes of the cycle, which favors the complete degradation of a fatty acid once the oxidation is initiated. Support has been given to this "all-or-none" thesis of Green by the recent series of reports by Chaikoff and his co-workers[26] on their studies of the sparing of fatty acid oxidation by carbohydrate. These workers have injected labeled fatty acids of various chain-length into rats and have found that injected acids of chain-length less than C_{12} are almost totally oxidized to $C^{14}O_2$ while the longer-chain acids (C_{14} to C_{18}) are largely stored as the C_{16} acid. This indicates that few if any short-chain acids escape the oxidative action of the acyl dehydrogenases and once attacked by these enzymes they continue the oxidative spiral to ultimate Krebs cycle oxidation. On the other hand, longer chain acids are more apt to be shortened or lengthened to 16-carbon chains and stored as such. As with the shorter acids however, once attached to a acyl dehydrogenase the only products are acetate or CO_2.

Synthesis--Although it had been postulated as early as 1907,[27] the first experimental demonstration that two carbon compounds are used in the synthesis of fatty acids was not reported until 1926. At this time Smedley-MacLean and Hoffert[28] demonstrated the accumulation of fatty acids (and sterols) in yeast cells grown in a medium containing either ethanol or sodium acetate as the sole carbon source.

The importance of two-carbon compounds in the biosynthesis of fat in animals was however not fully appreciated until after 1935 when Schoenheimer and Rittenberg[29] introduced the technique of isotopic labeling for the study of this problem. In 1942 Bloch and Rittenberg[30] reported that the small molecules from which fatty acids (and cholesterol) are synthesized in animals are acetic acid units, or a more reactive derivative of acetate.

Since 1942 the central role of acetate in the metabolism of fat has been well established, almost entirely on the basis of work carried out with the aid of isotopic tracers. One such study was the report by Popjak, et al.[31] in 1951 on the relationship between specific activity and chain-length of fatty acids from goat's milk following the intravenous injection of carboxyl-labeled C^{14}-acetate. By the stepwise splitting off of two carbon atoms as acetic acid from the carboxyl end of the recovered fatty acids, they were able to determine the origin of each of these two-carbon fragments. They concluded that fatty acids are synthesized by a process corresponding to a reversal of β-oxidation. This involves the condensation of two acetate molecules to form acetoacetic acid, which is then reduced, dehydrated and the double bond saturated to give butyric acid. This is followed by the addition of another acetate unit to the carboxyl end of the butyric acid to form the six carbon fatty acid, caproic acid, which then combines with another acetate unit to yield the eight-carbon caprylic acid, etc., up to palmitic acid.

The choice of milk fat for demonstration of this stepwise synthesis was a fortuitous experimental circumstance for nowhere else do the

intermediates of this synthesis occur in detectable amounts. Rittenberg and Bloch,[32] reporting on the in vivo synthesis of fatty acids from acetate in 1945 and Brady and Gurin,[33] in reporting this synthesis in vitro in 1950 found only uniformly labeled long-chain fatty acids and none of the intermediates as reported by Popjak. According to Popjak,[34] the glyceride fatty acids of milk represent metabolic products which have become stabilized by esterification with glycerol and secretion into the milk ducts. This process of secretion thus intervenes with great rapidity between the formation of these acids and their further metabolism in the cell, thereby trapping in the milk-glycerides substances which otherwise would be metabolized so rapidly as never to accumulate in detectable amounts. This rapid secretion, according to Popjak has only the effect of preserving the intermediary products of synthesis.

In support of Popjak's two-carbon unit synthesis, many reports have accumulated which demonstrate the occurence of stepwise two-carbon additions to the more readily studied longer-chain fatty acids. Stevens and Chaikoff[35] reported in 1951 the conversion of lauric (C_{12}) and myristic (C_{14}) acids to palmitic (C_{16}) and stearic (C_{18}) fatty acids by the addition of two-carbon units. In the same year, Zabin[36] demonstrated the conversion of palmitic acid to stearic acid by a two-carbon addition both in vivo and in vitro. This conversion had been studied earlier in vivo by Schoenheimer and Rittenberg[29] using deuterium-labeled fatty acids.

The isolation of enzyme systems capable of the β-oxidation of fatty acids described above proved to be the answer to the question of two-carbon unit synthesis of fatty acids, for all of these reactions are

reversible.[10] Thus it was possible to demonstrate the enzymatic synthesis of long-chain fatty acids by a reversal of the oxidative sequence previously illustrated. In fact, β-oxidation of a fatty acid provides not only the two-carbon building block for fatty acid synthesis but also a ready source of the energy for this synthesis since all of the acetate units will be present as acetyl-CoA. As with β-oxidation, the synthetic cycle is autocatalytic once initiated since the newly formed acid (Butyryl-CoA) retains its high energy bond and is prepared for the next condensation with another acetyl-CoA molecule.[37]

As pointed out by Green[25] the substrate affinities of these enzyme systems favors the formation of C_{16} or C_{18} fatty acids once the synthesis is initiated, which accounts for the absence in nature of the C_6 to C_{14} fatty acids (with the notable exception of milk fatty acids as pointed out above).

Cholesterol Metabolism

The transformation of labeled acetate into cholesterol in liver slices was first demonstrated in 1946 by Bloch, Borek and Rittenberg.[38] (Bloch and Rittenberg had previously reported this conversion in vivo.)[30] Since then, abundant confirmatory evidence has been reported from several laboratories.[39,40,41,42] Less is known, however, about the method of biosynthesis of cholesterol than the related synthesis of fatty acids from acetate. Fragments are slowly being pieced together to provide a more adequate understanding of this puzzle but at present comparatively little is known about the metabolic intermediates in this synthesis of cholesterol from acetate.

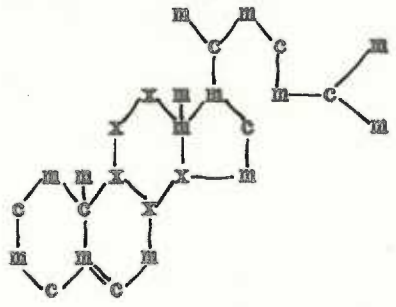
In 1950 Little and Bloch[43] studied the incorporation of both methyl and carboxyl labeled C^{14}-acetate, as well as acetate labeled with both C^{13} and C^{14}, into cholesterol. They were able to demonstrate the incorporation of methyl carbon of acetate into positions 18, 19, 26, 27, and probably 17, while the carbon atom 25 and probably 10 were shown to arise from the acetate carboxyl atom. They also found the ratio of methyl to carboxyl derived carbons in cholesterol to be 1.27. This was shown to be due to the greater incorporation of carboxyl carbons into the nucleus of the cholesterol molecule than into the iso-octyl side chain. The ratio of methyl/carboxyl groups incorporated into the nucleus was calculated to be 1.1 while the corresponding value for the side chain was 1.67.

It should be pointed out that contrary to earlier assumptions, acetate is apparently not the only precursor utilized directly by mammalian tissue for the biosynthesis of cholesterol. At the present time, acetate,[38] acetone,[44] pyruvate,[45] butyrate, hexanoate and octanoate,[33] as well as the isopropyl fragment of isovalerate[46] have been shown to be incorporated directly into cholesterol by rat liver slices without first being converted to acetate. That acetate is, nevertheless, a primary carbon source for the biosynthesis of cholesterol, as well as of fatty acids, appears to be well documented.[30,47]

At the time this discovery was made, no biochemical reactions were known which would account for the formation of this complex sterol structure from such a simple substance as acetic acid. Only within the past five years has any significant order been achieved from the array of conflicting information, largely through the work of Dr. Konrad Bloch.

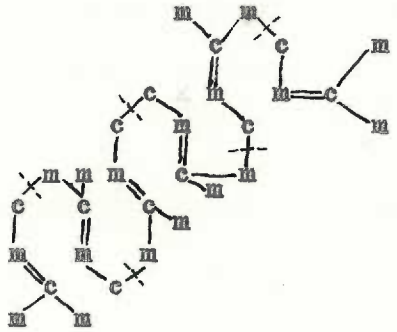
Prior to the advent of chemical degradation procedures applicable to this sterol, significant advances in the understanding of the biosynthesis of the molecule were hindered since it was impossible to identify the labeled positions arising from one or the other of the carbon atoms of acetate. In addition, such procedures as were developed required large amounts of labeled cholesterol for the extensive chemical manipulations required. The demonstration of in vitro synthesis of cholesterol from labeled acetate in liver slices by Bloch, Borek and Rittenberg[38] in 1946 made available for the first time a biosynthesized preparation of sufficiently high isotope content to allow dilution with non-labeled carrier cholesterol without loss of accuracy. Practically all degradations of cholesterol biosynthesized from labeled precursors have been carried out on material obtained from liver slices.[48]

The results of many degradation experiments, utilizing cholesterol biosynthesized from labeled acetate were summarized in 1955 by Popjak,[48] who presented the following composite picture, where m are methyl and c are carboxyl carbons of acetate:

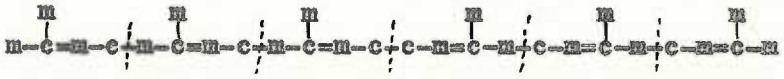


Carbon atoms 8, 9, 11, 12 and 14 had not been characterized at that time but atoms 8 and 12 have since been found to be methyl carbons. The origin of atoms 9, 11, and 14 has yet to be determined.

Largely as a result of studies on synthetic rubber polymerization by Bonner and Arreguin[49] in 1949, Bloch[50] had proposed a theoretical scheme for the distribution of acetate carbons in cholesterol, based on the cyclization of squalene, which was entirely consistent with Popjak's summary of degradation studies reported above. This theoretical scheme was suggested to Bloch by the acetate carbon distribution found in the isopentane skeleton ("isoprenoid unit") from which Bonner and Arreguin[49] had been able to synthesize rubber. Bloch recognized this acetate carbon distribution as being compatable with the theory postulated in 1932 by Robinson[51] in which the triterpenoid hydrocarbon squalene, composed of six of these isoprenoid units was considered an intermediate in the biosynthesis of cholesterol. The application of Bonner and Arreguin's reported distribution of acetate carbons in isoprenoid units to these same units in squalene led to Bloch's proposed distribution of acetate carbons in cholesterol, which would result from the folding and cyclization of squalene. This hypothesis required the migration of one methyl group from position 8 (or 14) to position 13 when the cyclization of the hydrocarbon takes place. This folded squalene molecule would have the following structure (m = acetate methyl carbon; c = acetate carboxyl carbon; isoprenoid units included between the dotted lines):



Bloch[52] provided experimental support for his hypothesis in 1953 when he demonstrated that squalene could be synthesized from acetate in the rat when the rat is fed squalene and labeled acetate simultaneously. Feeding of this biosynthesized squalene to mice resulted in its conversion into cholesterol much more efficiently than any precursor previously tried. These observations have since been confirmed by others.[53,54] The biosynthesis of squalene sufficiently labeled to allow chemical degradation and identification of the acetate carbon positions was accomplished by Popjak[55] in 1954 and was found to be in complete agreement with Bloch's[50] earlier hypothesis. The schamatic formula of squalene showing the distribution of acetate carbons as demonstrated by Popjak is as follows (m = methyl; c = carboxyl; dotted line = isoprenoid unit):



Further illucidation of the origin of these isoprenoid units has recently been presented. In 1954 Rudney[56] reported the isotope distribution in both β-hydroxy-β-methylgluconic and β,β-dimethylacrylic acids synthesized from C^{14}-acetate and found the isotope distribution in both acids to be in accordance with the known distribution in the isoprenoid units of squalene. He envisaged the biosynthesis of isoprenoid units to proceed by a condensation of acetoacetyl-CoA with acetyl-CoA in a manner analogous to the formation of citrate from oxalacetate and acetyl-CoA. Dehydration and decarboxylation of this condensation product would yield β,β-dimethylacrylic acid in which the acetate carbons are distributed according to the pattern of isoprenoid units.

In summary, it has been established that cholesterol is synthesized entirely from two-carbon acetyl-CoA units, probably along the following pathway:

A). 2 acetyl-CoA ⟶ acetoacetyl-CoA

B). acetoacetyl-CoA + acetyl-CoA ⟶ β-hydroxy-β-methylglutaryl-CoA

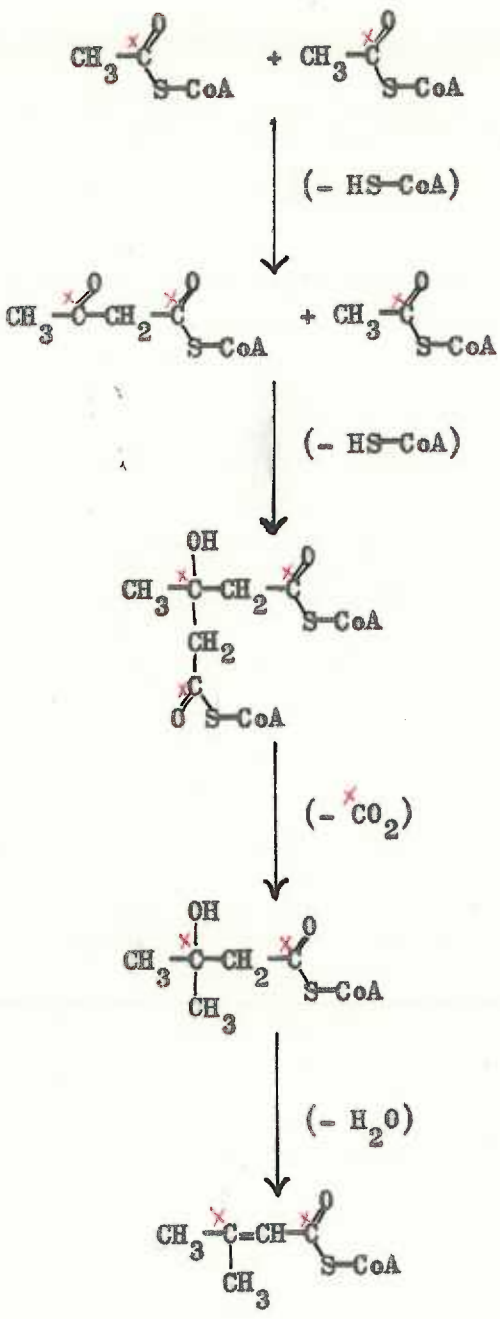
C). β-hydroxy-β-methylglutaryl-CoA ⟶ β-hydroxy-β-methylbutyryl-CoA + CO_2

D). β-hydroxy-β-methylbutyryl-CoA ⟶ β,β-dimethylacrilyl-CoA + H_2O

E). 6 β,β-dimethylacrilyl-CoA $\xrightarrow{?}$ squalene

F). squalene $\xrightarrow{?}$ cholesterol + 3 methyl groups

The method by which acetate condenses to yield the "isoprenoid unit" with the acetate carbon distribution porposed by Bloch may be diagrammed as follows on the next page[57] (red x indicates acetate carboxyl carbon):



A). 2 acetyl-CoA $\xrightarrow{-\text{HS-CoA}}$

B). acetoacetyl-CoA + acetyl-CoA $\xrightarrow{-\text{HS-CoA}}$

C). β-hydroxy-β-methylglutaryl-CoA $\xrightarrow{-CO_2}$
(HMG)

D). β-hydroxy-β-methylbutyryl-CoA $\xrightarrow{-H_2O}$
(β-hydroxyisovaleryl-CoA; HIV)

E). β,β-dimethylacrilyl-CoA
("Isoprenoid Unit")
(β-methylcrotonyl-CoA; senecioyl-CoA; DMA)

Summary

A number of workers have established that acetate is the primary building unit from which animals synthesize fatty acids and cholesterol,[58] but the manner in which the acetate molecules condense to form these substances was not known with certainty until recently in the case of fatty acids and our knowledge of cholesterol is still sketchy. Based on the foregoing discussion, a general scheme of acetate metabolism is given in Figure 1. Although not directly related to lipogenesis, the role of acetate in the tricarboxylic acid cycle is included because of its importance both in supplying the energy requirements of lipid synthesis, and in the oxidation of acetate units derived from fatty acid oxidation.

Figure 1

Scheme of Acetate Metabolism

A simplified outline of the three major metabolic pathways of acetate in the body: 1) oxidation to CO_2 and water via the Krebs cycle; 2) synthesis of fatty acids; 3) synthesis of cholesterol.

SCHEME OF ACETATE METABOLISM

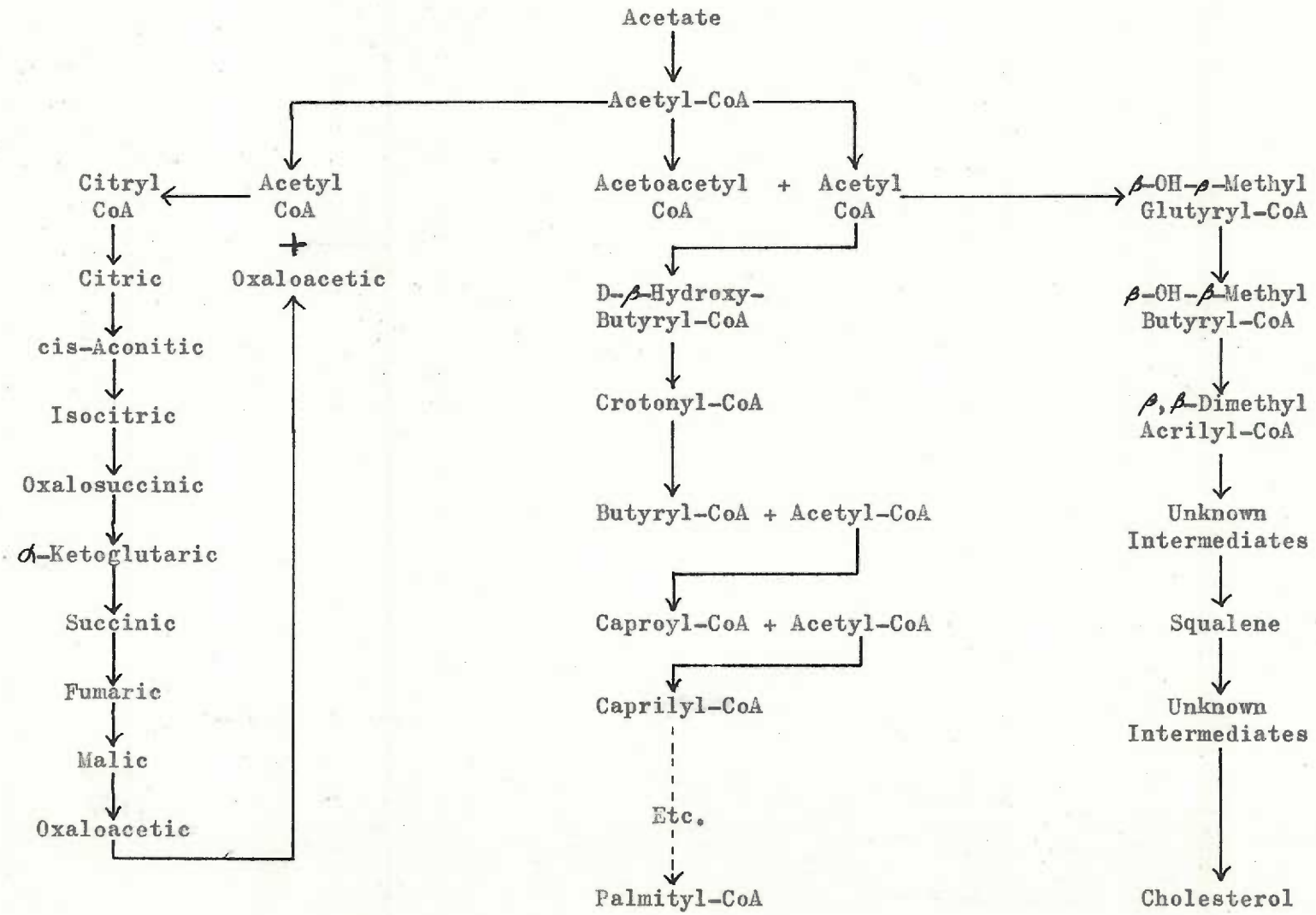


Figure 1

CHAPTER II

MATERIALS AND TECHNIQUES

Animal Preparation

Source of Animals

The animals used in these studies were albino rats of the Sprague-Dawley strain. All were young adult males weighing 200 to 250 gm. at the time of sacrifice. About one half of the animals were obtained from the Sprague-Dawley Rat Farm at Madison, Wisconsin, and the remainder from the Northwest Rodent Co. at Pullman, Washington, and the colony of Sprague-Dawley Strain Rats maintained by the University of Oregon Medical School Department of Biochemistry.

The rats weighed between 125 and 175 gm. when obtained and were maintained in a cage-hood assembly developed in this laboratory.[59] We have observed the most satisfactory weight gains when two to three rats are maintained in a standard 8" x 8" x 16" wire cage and this maintained in the cage-hood assembly.

Trained-Feeding

Controlled feeding was, in most cases, started at the time the rats were received. The controlled feeding regime consisted of two daily feedings of ten grams of Purina Laboratory Chow per animal. The chow was placed in the cage at 12 hour intervals and, at the end of one hour, any food remaining in the cage was removed. Water was

allowed ad libitum and the rats were weighed daily prior to the morning feeding.

Four to six days of this regime was sufficient to train the rats to eat the entire ten grams almost immediately and rarely was it necessary to remove any food from a cage after the first week of controlled feeding.

After an initial five to ten gram weight loss while the rats adjusted to the regime, they would show a consistent daily weight gain of four to six grams a day. All rats used in these experiments had been train-fed for at least one week and had shown a constant weight gain for at least three days prior to use. For a more detailed discussion of train-feeding, refer to The Influence of Refeeding upon Lipogenesis in the Intact Fasted Rat.[60]

Fasting

Since these studies were designed to supplement and extend the in vivo studies previously reported from this laboratory,[61,62,63,64] it is desirable to adhere as closely as possible to the definitions used in these reports. Unfortunately our current definition of fasting periods is not entirely adequate for in vitro discussions and a modified nomenclature has therefore been adopted.

Two factors were considered in arriving at the definition of fasting. First, since these rats had been trained to consume ten grams of chow within one hour, this first one hour period after the food is placed in the cage will be defined as the feeding period. Thus an animal sacrificed 60 min. after food is placed in the cage is termed a one hour fed rat. Second, since any food remaining in the cage at

the end of one hour is removed, this interval between one hour after food is placed in the cage until eleven hours later when the animals would normally be fed again is termed the fast period. Thus, if an animal is used two hours after food was placed in the cage or one hour after the remaining food was removed it is termed a one hour fasted rat.

Two fallacies in this nomenclature are immediately apparent but unavoidable. First, since the rats often learn to eat the entire ten grams of chow in less than 30 min., this entire one hour period does not represent a period of continued food intake. Second, as will be described below, there is a one hour interval between sacrifice of the rat and the time acetate-C^{14} is added to the substrate containing the liver slices. Since the slices are deprived of normal circulating metabolites during this period it might be considered one hour of fasting. Although this one hour interval is constant in all experiments it assumes greater significance in the short term fasted animal, (as the 30 min. fed rat where it contributes an appreciable period of food deprivation), than it does in the 12 hour fasted rat where it may be considered insignificant. For the above reasons, a true "short-term fast" as described in previous in vivo studies from this laboratory[60] cannot be duplicated in in vitro studies, since the shortest possible fasting period must include this 60 min. period of metabolite deprivation.

Although liver glycogen levels may be used as an index of the "fed" or "fasted" condition, the dramatic responses of lipogenic processes previously reported from this laboratory[60] occur very shortly after feeding and prior to the deposition of appreciable amounts of hepatic

glycogen. For this reason, no attempt was made in the present studies to measure this metabolic reserve.

Slice Preparation

Several techniques for the preparation of liver slices were tried in an attempt to obtain metabolically active slices which would conform to the conditions of thickness, uniformity of thickness and cellular integrity as outlined in Chapter I.

In our initial experiments, slices were prepared free-hand using a 4 cm x 6 cm glass plate with a longitudinal groove 0.5 mm deep by 2.5 cm wide and a double edge razor blade. The block of tissue was held against the glass plate and the blade passed between the block of liver and the glass plate, thus slicing off only that portion of liver which had been pressed into the 0.5 mm groove in the glass plate. Although this technique provided fairly active slices, it was found to be too time consuming and the slices lacked uniformity of thickness.

In order to obtain slices more rapidly, an attempt was made to use the freezing microtome. A small block of liver was placed on the microtome and quickly frozen with a blast of compressed CO_2. Slices were then cut as rapidly as possible and transferred to a solution of Krebs-Ringer-Phosphate buffer maintained at $4^{\circ}C$. This technique was quickly discarded when it was found that even the brief period of freezing employed (less than ten seconds per block of tissue) caused complete inactivation of respiration in the slices.

Our present slicing technique, described below, utilizes the commercially available Stadie-Riggs microtome* (Figure 2). Using this apparatus, four grams of slices of uniform thickness averaging 0.5 mm can be cut within 15 min. with minimal trauma to the slice.

Technique of Slicing

Animals prepared as previously described are killed by decapitation and the liver quickly removed as atraumatically as possible by cutting it free of its ligamentous attachments and then sectioning the portal vein and hepatic artery and vein. The organ is transferred to a crystalizing dish containing iced buffer.

A block of tissue of convenient size, generally about one cm square, is cut from the liver and placed on a disc of moistened filter paper on the microtome. A few drops of buffer are put on the tissue block; the microtome and blade are lubricated with a small quantity of buffer and slices quickly cut with a to-and-fro motion of the blade. We have found it convenient to leave the thumb-screws on the microtome loose and hold the two parts of the microtome together by holding the lower part between the thumb and the third, fourth, and fifth fingers of the left hand and providing the necessary pressure on the upper part (B) with the index finger of the same hand. The entire left hand then provides the slight amount of pressure on the two parts together (A-B) which is required to compress a portion of the tissue block into the 0.5 mm indentation in the upper part (A) of the microtome. Using this technique, the two parts of the microtome (A-B and C) can be separated slightly after the

*Arthur H. Thomas Company, Philadelphia

Figure 2

The Stadie-Riggs Microtome

See text for description.

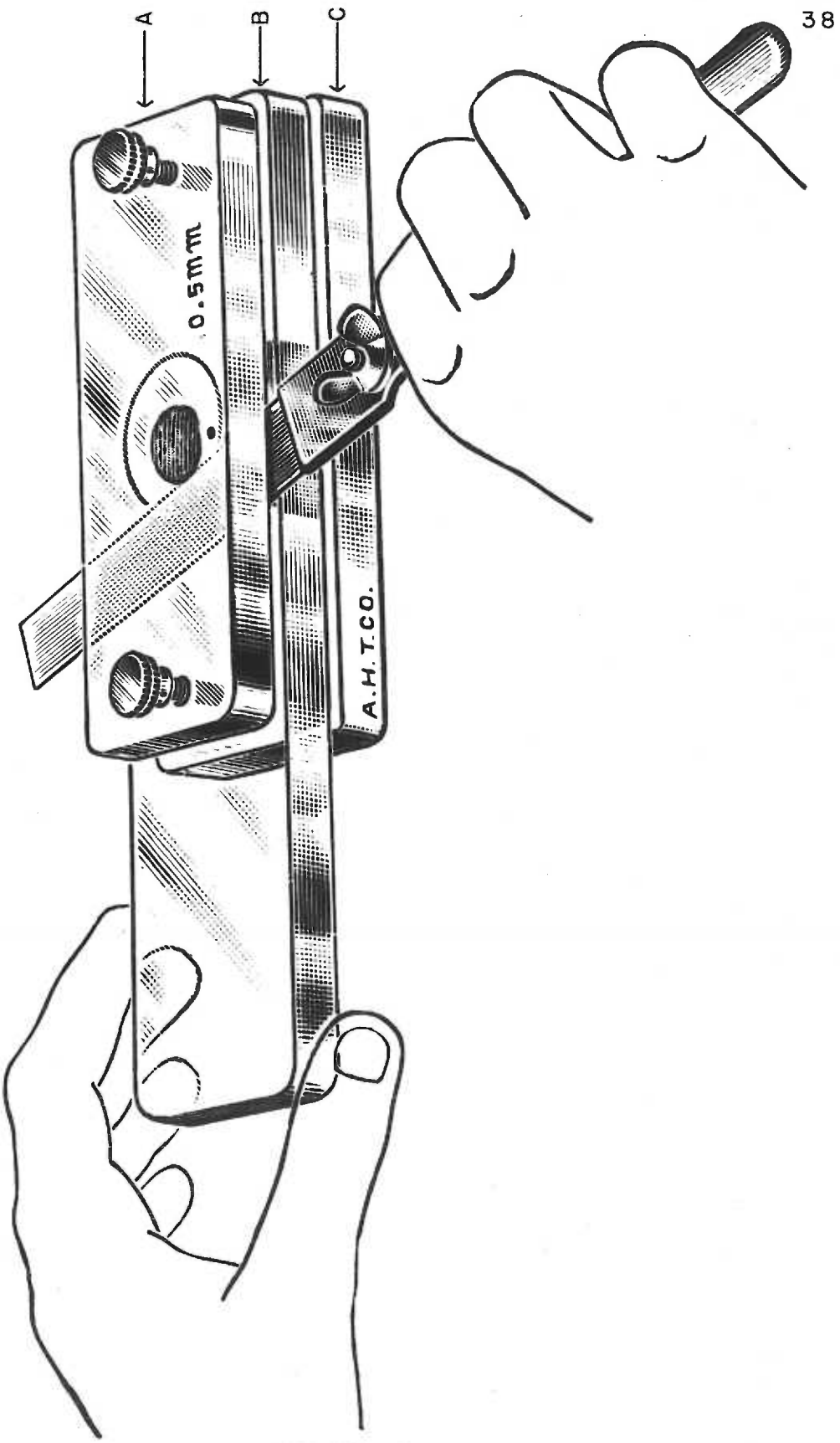


FIGURE 2

slice has been cut and the slice will adhere to the blade which is then withdrawn and the slice removed from the blade with a small pair of thumb forceps. This eliminates the necessity of floating the slice off the inverted slicer as suggested by Stadie.[65]

Since the first slice from each block of tissue is composed partially of liver capsule, it is discarded. Subsequent slices are distributed to four or more 2.5 by 5 cm flat bottom vials prepared prior to decapitation of the rat so as to contain a weighed quantity of iced buffer as follows: five ml of buffer solution is measured into the first vial, which is then weighed, and to the other vials is added buffer to make each of their weights equal ($\pm$ 0.005 gm.) to that of the first. In this manner, the weight of slices may be obtained by subtracting this tare weight of the vial plus the buffer from the gross weight after the slices have been added to the vial. These vials are kept in a tray of ice during slicing to maintain the slices at about 8°C.

In early experiments, each vial in turn was filled with slices obtained from the microtome. Thus, the first vial contained the first gram of slices cut, the second vial contained the second gram cut, etc. Oxygen utilization measurements from these slices showed that the first gram of slices had the highest oxygen utilization, the second gram had the second highest oxygen utilization, etc. This difference was apparently due to the fact that the slices obtained last had been maintained in a relatively hypoxic condition in the uncut liver one to three times longer than slices cut earlier. This assumption was confirmed when the slices were randomized among all four vials by placing the first slice in the first vial, the second slice in the second vial, etc. In

this manner, each vial contained slices from each block of tissue. Oxygen utilization measurements from these slices were essentially identical (Figure 3).

Transfer of Slices to Warburg Flasks

The vials containing the slices were weighed and the slices transferred to Warburg flasks with the aid of a small funnel. The vial and funnel were rinsed with a 20 ml portion of buffer previously measured into a test tube and stored in a beaker of cracked ice.

Following transfer of the slices to the Warburg flasks, the flasks were individually flushed with 100% oxygen for one min., the gas flowing at a rate of six liters per min. through a 6 mm delivery tube. After oxygen flushing, the CO_2 absorber tube described below was placed in the center well of the flask.

The Warburg flasks, containing the slices and CO_2 absorber, were quickly attached to the manometer and placed in the constant temperature bath for 30 min. of thermal equilibration before tipping in the acetate-C^{14} from the flask sidearm. Total time from decapitation of the rat until the beginning of thermal equilibration was usually 28 $\pm$2 min.

Incubation

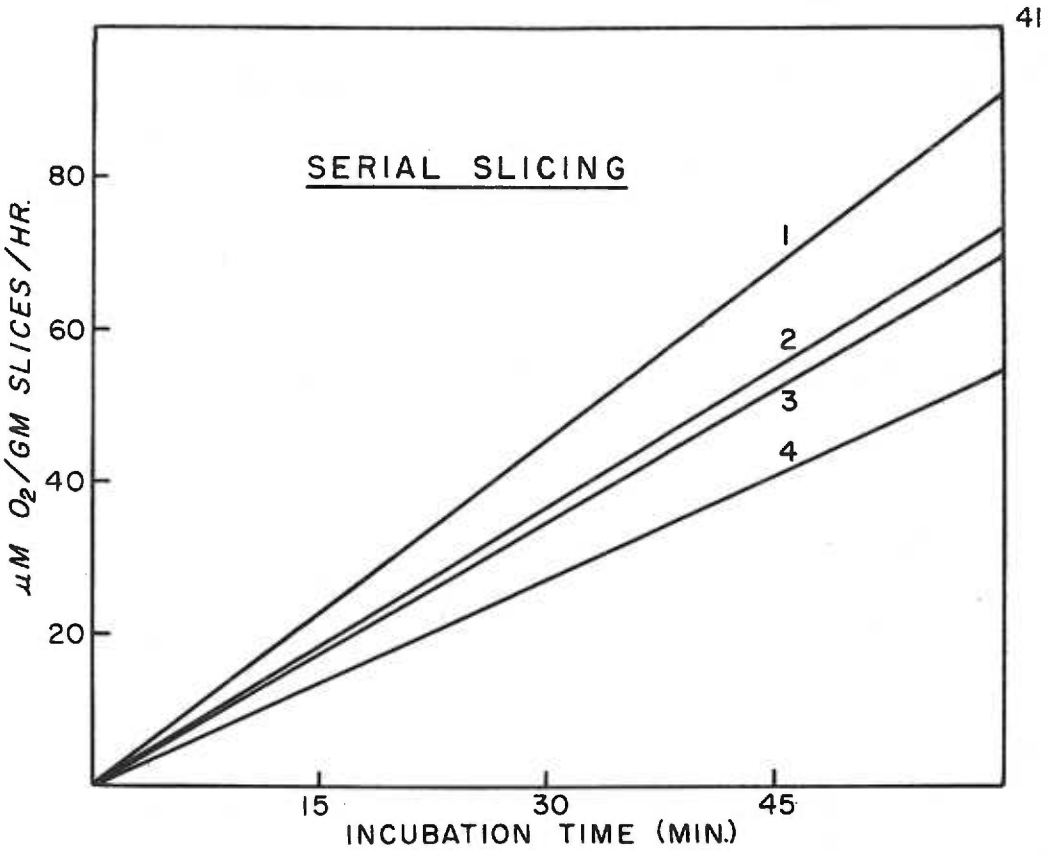
Incubation Flasks

Custom made, double sidearm, Warburg type, reaction flasks obtained from Machlett Co. were used in these studies. These flasks had a capacity of 125 ml and had two sidearms of about 5 ml capacity each for the addition of substances to the suspending medium. The flasks were fitted

Figure 3

The Influence of Random Slicing on O_2 Utilization by Rat Liver Slices

The upper figure shows the spread of values obtained when the first gram of slices cut are placed in flask number 1, the second gram in flask number 2, etc. The lower figure shows the essentially uniform O_2 uptake between flasks when the slices are randomized by placing successive slices in each of the four flasks so that each flask contains slices obtained from the beginning to the end of the slicing procedure. O_2 uptake, in uM per gram of liver slices, is shown for the first hour of incubation for each of the 4 flasks comprising a single experiment.


SERIAL SLICING
1
2
3
4
80
60
40
20
μM O2/GM SLICES/HR.
15
30
45
INCUBATION TIME (MIN.)

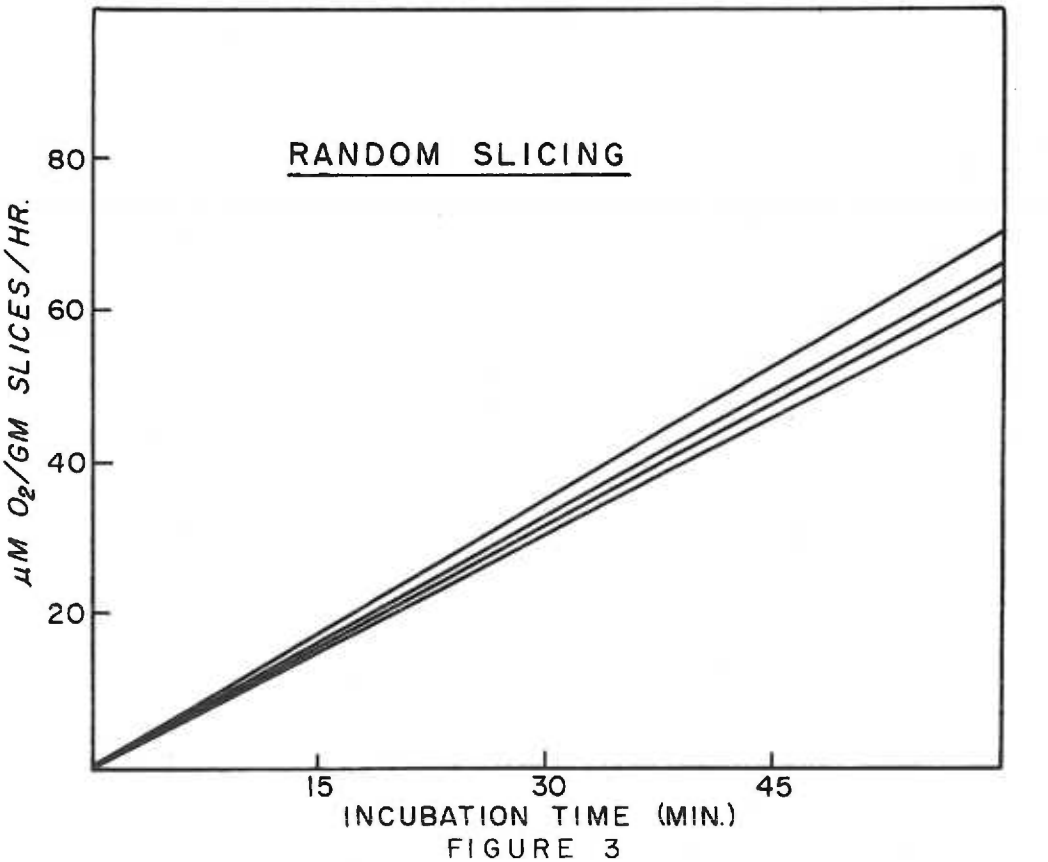

RANDOM SLICING
80
60
40
20
μM O2/GM SLICES/HR.
15
30
45
INCUBATION TIME (MIN.)

FIGURE 3

with a female ₹ 16/20 joint for attaching to the manometer and each of the sidearms was fitted at the top with a glass stoppered ₹ 7/15 joint.

Buffer Preparation

Krebs-Ringer phosphate buffer, pH 7.4 was used for all experiments and was prepared according to the method of Umbreit, Burris and Stauffer, described in the monograph "Manometric Techniques and Tissue Metabolism".[4] The final solution was chilled and gassed with 100% oxygen for ten min. Fresh buffer was prepared for each experiment from the concentrated solutions which were stored in the refrigerator. Once prepared, the buffer was kept chilled and was used in less than 8 hrs.

CO_2 Absorption

CO_2 of the gas phase of the flask was continually absorbed by NaOH absorbers which were prepared in advance by placing a roll of Whatman No. 50 filter paper in a small test tube selected to fit concentrically inside the Warburg flask center well. The tube was cut to a length slightly longer than the center well and flared slightly at the top to facilitate grasping it with a pair of rat-tooth thumb forceps and to prevent creeping of the NaOH. 0.2 ml of 8.7 N NaOH was used to moisten the filter paper in the tube. This tube with its moistened filter paper was stored in a sealed jar containing a saturated solution of NaOH until transferred to the Warburg flask. At the end of incubation, 0.5 ml of 12.5 N H_2SO_4 was added to the substrate by means of a syringe and needle introduced through one of the sidearms in order to stop the metabolic reactions of the slices and to liberate CO_2 contained in the

tissue slice and the liquid phase of the flask. The flask was then replaced in the bath for 30 min. to allow liberation of all CO_2 and its absorption on the filter paper.

Acetate Addition

All acetate-C^{14} used in these experiments was obtained from the same lot of sodium acetate-1-C^{14} which was prepared in this laboratory as previously described.[66] This acetate, number 104, in a volume of 25 ml, contained about 20 uC per ml or 2.5×10^6 cpm per ml as an infinitely thick $BaCO_3$ plate counted in a windowless gas flow counter. One ml of this solution was found to contain 6.33 uM of acetate when steam-distilled and titrated. This stock solution was kept frozen in a narrow-mouth, rubber-stoppered centrifuge tube and 1 ml of this solution was diluted to 25 ml with distilled water as needed to provide a useable dilution of acetate-C^{14} for addition to the liver slices. This 1 to 25 dilution of the original stock solution was labeled #104-A, #104-B, etc., as new dilutions were prepared and contained about 100,000 cpm in 0.25 uM of sodium acetate per ml.

In most experiments, one ml of this 1 to 25 dilution was added to the sidearm of each flask prior to transferring the slices into the flask and was tipped into the substrate exactly one hour after the animal had been killed or about 30 min. after the flasks containing the slices had been placed in the constant temperature bath. After tipping the acetate into the substrate the sidearm was washed three times by tipping the substrate into the sidearm and then tipping it back into the flask. The entire label addition procedure required less than 30 sec. per flask.

Oxygen Utilization

Two min. after the acetate was tipped into the substrate the manometer was adjusted to the 15 cm mark and the stopcock closed. This was considered the zero time for oxygen measurements. Manometer readings were made every 15 min. thereafter for the duration of the incubation. The thermobarometer readings were taken 15 sec. before each oxygen reading and appropriate corrections made in the observed manometer readings.

In order to provide equal incubation periods, acetate was added to the substrates at two min. intervals. For this reason, there was a two min. interval between the manometer readings for each successive flask. Since the acid was added to the substrate of successive flasks at two min. intervals, the total incubation time for each flask was held equal.

Tissue Fractionation and Assay

CO_2 Transfer and Plating

Thirty min. after the contents of the flasks were acidified, the flask was removed from the bath, quickly removed from the manometer, and stoppered briefly until the CO_2 absorber was removed from the flask. After removal of the absorber tube, the filter paper roll was removed from the tube with a pair of rat-tooth thumb forceps and transferred to a 50 ml thick-walled centrifuge tube. The absorber tube was then washed five times with hot, CO_2-free, distilled water, the washings being added to the centrifuge tube containing the paper roll. The tube was then sealed with a tight-fitting rubber vial closure and

allowed to stand until the filter paper roll was removed (see below). The effectiveness of the rubber closure was easily confirmed by the presence of a vacuum in the tube when it had cooled to room temperature. At a convenient time, usually the following day, the tube was opened and the filter paper strip was grasped at one corner with a small hemostat and held over the tube while hot, CO_2-free, distilled water was allowed to drip on it until the total volume in the centrifuge tube was about 40 ml. About ten ml of this volume was contributed by the five washings from the CO_2 absorber tube and the remaining 30 ml was the quantity of water used to elute the NaOH and Na_2CO_3 from the filter paper strip. 0.5 ml of 0.498 M Na_2CO_3 was added to the tube to serve as carrier and the carbonate precipitated as $BaCO_3$ and plated by our usual technique.[67]

Control flasks, containing buffer, CO_2 absorber and acetate-C^{14} were run repeatedly and never contained more than two uM of CO_2 after one hour of incubation and the transfer and plating procedure described above. This amount of CO_2 represents only about 2% of the total CO_2 found at the end of one hour of incubation. These control samples never counted more than five cpm above background.

Slice Transfer and Saponification

After removal of the CO_2 absorber, the substrate was decanted off the slices and the slices were then transferred with a small spatula to a 50 ml screw-cap culture tube. These slices were then washed, with swirling, using four 10 ml portions of water and the washings added to the substrate. The tube containing the substrate and the washings was stoppered and frozen until it was analyzed.

Twenty ml of 2 M KOH in 95% alcohol was added to the tube containing the washed slices and the tube was covered with a large glass marble. It was then placed on a steam bath and allowed to reflux slowly for two hours. Additional alcohol was added as necessary to maintain the volume.

At the end of two hours, the marble was removed and all alcohol blown off. Four ml of alcohol was then added back to the tube and the volume taken to 20 ml with water, giving a 20% concentration of alcohol.

Lipid Extraction

All extraction procedures were carried out in the 50 ml screw-cap culture tube into which the slices were initially transferred, washed, and later saponified, thus eliminating at least two transfers which would have been necessary if the usual separatory funnel extraction technique had been used.

The significance of minimal transfers and solvent volumes can be appreciated in light of the small quantities of lipid present in one gram of liver, these being about 25 mg. of fatty acid and two mg. of cholesterol per gram of tissue. Our technique of carrying out all procedures in one vessel minimizes losses which would be unavoidable by more bulky methods employing several transfers to boiling flasks and separatory funnels and has enabled us to obtain extremely reproduceable recoveries.

In order to provide the gas-tight seal necessary for extraction with low-boiling solvents, the cork liners supplied with the screw caps on the culture tubes were removed and replaced with discs cut from 1/16 inch polyethylene sheets.

Thirty ml of low-boiling petroleum ether (B. P. 30° -60°C) was added to the tube containing the saponified slices dissolved in 20 ml of the water-alcohol solution. The cap was screwed on securely and tested for leaks by shaking a few times by hand. Several such tubes were then placed in a horizontal position on a shaking machine and shaken for one minute at a rate of 280, 1½ in. strokes/min. The tubes were then allowed to stand in a vertical position about five min. until complete separation h a d occured. The cap was then removed and the petroleum ether layer aspirated with a 30 ml syringe equipped with a four-ince, 18 guage, blunted needle. The aspirated petroleum ether was transferred to a 250 ml wide mouth erlenmeyer flask containing a quantity of anhydrous sodium sulfate sufficient to layer the bottom of the flask.

Five such extractions were performed to remove the non-saponifiable lipid. The contents of the culture tube were then acidified with concentrated HCl (to congo red paper) and cooled to room temperature. Five more extractions with petroleum ether were performed to remove the saponifiable lipid, the petroleum ether again being transferred to flasks containing sodium sulfate.

The two lipid fractions were allowed to stand over sodium sulfate over night and then filtered into 500 ml flat-bottom boiling flasks, using five 50 ml petroleum ether washes. The petroleum ether was then evaporated almost to dryness on a steam bath, removed from the bath, and taken to dryness with a stream of dry nitrogen.

Four ml of alcohol was then added to the flasks containing the non-saponifiable lipid fraction (mainly cholesterol) and five ml of acetone was added to the flasks containing the saponifiable lipid fraction (mainly fatty acids). The flasks were then returned to the steam bath and warmed until the refluxing column of solvent just reached the mouth of the flask when they were removed from the bath and allowed to return to room temperature.

The cooled solvents were then transferred to appropriate containers with the aid of volumetric pipettes. The solvents were aspirated into the pipettes just to the bulb, thereby avoiding loss of lipid in the expanded portion of the pipettes. This process of refluxing, cooling and transferring was repeated five times, using a total volume of 20 ml of alcohol for the non-saponifiable fraction and 25 ml of acetone for the saponifiable fraction.

The non-saponifiable fraction was transferred without filtering to 25 ml volumetric flasks. The saponifiable fraction was transferred to tared 15 by 40 mm flat-bottom shell vials supported in a 70°C water-bath. As each five ml volume of acetone was transferred to the shell vial it was blown off with a stream of dry nitrogen, thus allowing several transfers into these small, light weight vials.

Radioassay

Color-reacting sterols in the non-saponifiable fraction (mainly cholesterol) were assayed by the colorimetric method of Zlatkis.[68] The saponifiable fraction was dried in a vacuum desicator overnight and then weighed.

In our initial experiments both cholesterol and fatty acids were combusted by the Van Slyke wet combustion technique and the CO_2 precipitated as barium carbonate and plated by our usual technique.[69] More recently we have abandoned the time-consuming wet combustion technique in favor of the direct plate method of radioassay. Aliquots of the cholesterol solutions (usually 0.5 ml) are pipetted into 5 cm^2 aluminum cups and the alcohol allowed to evaporate undisturbed at room temperature. The cups are then dried for one hour at 105°C and counted under a windowless gas-flow counter as infinitely thin plates. Samples of cholesterol so prepared must contain less than 0.3 mg. of cholesterol per 5 cm^2 to be regarded as infinitely thin.[70] The fatty acids are transferred with a small spatula to a tared aluminum planchet on which has been inscribed a 5 cm^2 circle. The lipid is spread out in a thin layer with the spatula to cover the inscribed circle and then warmed under an infrared lamp to allow it to flow into an even layer. The planchet is then placed briefly on a cube of ice to quickly resolidify the fatty acid. The planchet is then reweighed and the radioactivity assayed as for cholesterol. We have been able to direct plate five to ten mg. of fatty acid on a planchet with good reproducability by this technique. The specific activity of the cholesterol samples is determined as follows:

$$SA = \text{cpm/mg. cholesterol} = \frac{\text{total cpm - background}}{\text{mg. of cholesterol in cup.}}$$

This specific activity figure may be converted to its infinitely thick $BaCO_3$ plate equivalent by dividing by a factor of 10.0.* Fatty acid

*This factor was experimentally determined in this laboratory and was found to be applicable for all cups containing less than 0.3 mg. of cholesterol per cup ("infinitely thin").

specific activity is calculated as follows:

$$\frac{\text{total counts/min/plate-bkg}}{g} \times 100 = \text{counts/min/mg. fatty acid} = SA.$$

The g factors used for the conversion of these fatty acid specific activities to equivalent activity as infinitely thick barium carbonate were determined experimentally in this laboratory.

CHAPTER III

RESULTS AND DISCUSSION

Preliminary Studies

Since tissue slice studies had not been performed previously in this laboratory, it was felt necessary to precede any actual metabolic studies with experiments which would establish the viability of our slice preparation. This was accomplished by studying the respiration of a number of flasks containing rat liver slices in a Krebs-Ringer-Phosphate buffered medium. In the first two such experiments, 16 flasks containing 0.5 to 0.8 gm. of slices each, obtained from two animals, were used. O_2 utilization ranged from 31 to 85 uM O_2 per gm. wet weight of slices per hour with an average of 66 uM/gm./hr. This value corresponds closely to the value of 61 uM/gm./hr reported by Field, et al. in their study of tissue respiration in the rat.[71] CO_2 recoveries ranged from 36 to 74 uM/gm./hr with an average of 53 uM/gm./hr. These averages give an RQ (CO_2/O_2) of 0.81 which is compatable with the nutritional state of the animals used. It is interesting that O_2 utilization was usually well maintained up to three hours with a maximum decrease of 17% over the entire three hour period while Field, et al. report a 29% decrease in O_2 utilization in the first hour of incubation. In most cases, we have actually observed a small increase in respiratory rate during the first hour of incubation amounting to 5 to 10% above the respiratory rate for the first 15 min. of incubation.

Time Course of Incubation

Introduction

It is apparent that several factors are involved in determining the metabolic status of a liver slice preparation at any given time from the sacrifice of the animal until all metabolic activity has ceased. Initially, the nutritional state of the animal will determine both the character of the metabolic processes taking place in the liver (i.e., predominately synthetic or oxidative) and the amount of metabolic reserve present (primarily as glycogen). This metabolic activity is subsequently modified by the conditions of slice preparation, composition of the suspending medium, composition of added substrate, composition and availability of the gas phase, temperature of incubation, and finally, by the duration of the incubation. The integration of all of these factors will conspire to determine the qualitative and quantitative status of the surviving tissue at any given time of incubation. Because of this, it is important to determine the time or length of incubation at which the reactions to be studied are proceeding at measurable as well as fairly stable rates.

Methods

Since previous reports from this laboratory had indicated that hepatic fatty acid synthesis in vivo was maximal about one hour postprandially,[60,61,63] it was decided to use one hour fasted animals in in vitro incubation studies. Since there is a one hour delay from sacrifice of the rat until the acetate-1-C^{14} addition, this may be considered as an hour of fasting as far as the liver slices are concerned. For this reason, it was felt that slices obtained from an

animal sacrificed immediately following the one hour eating period would probably more nearly reflect the metabolic status of intact liver tissue in the one hour fasted rat used for in vivo studies. To avoid confusion, these animals were called "zero-fasted" or "60 min.-fed" as per the standard nomenclature in this laboratory even though it was felt that the slices so obtained had been deprived of circulatory metabolites for one hour prior to labeling with acetate-1-C^{14} and would be the equivalent of one hour fasted rats used in vitro. In this initial group of experiments, seven animals were used. All animals were young adult male Wisconsin Sprague-Dawley rats carefully trained to feeding and sacrificed by decapitation immediately following the standard one hour feeding period. Slice preparation was as previously described and 1 ml of an acetate-1-C^{14} solution containing 100,000 cpm in 0.253 uM Na acetate was added to each flask. Serial flasks were removed from the incubation bath at 30 min., 60 min., 90 min. and 120 min. following addition of the acetate-1-C^{14}, and the tissue "killed" by acidification.

Results and Discussion

Table I presents the O_2 utilization and CO_2 recovery data for these animals. O_2 utilization is seen to have increased from 52.4 uM/gm./hr to 68.2 uM/gm./hr, a 23% increase in the rate of O_2 utilization during the two hour incubation period. During this same interval, CO_2 production has decreased from 67.8 to 60.2 uM/gm./hr, an 11.2% decrease. This increase in O_2 utilization and decrease in CO_2 production results in a 32% decrease in the RQ (CO_2/O_2). This decrease in RQ from values near unity to 0.7 is indicative of the nutritional status of these animals prior to sacrifice as well as the absence of organic metabolites in the

Table I

TIME COURSE OF INCUBATION

O_2 and CO_2

uM/Gm./Hr

	Min of Incubation							
	30 min		60 min		90 min		120 min	
	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2
1)	65.6	63.8	44.5	51.8				
2)	51.0	70.1	63.3	55.6	28.2	45.0	62.2	45.4
3)	36.8	66.4	33.3	78.0	57.9	53.6	59.7	60.2
4)	65.4		50.6		71.3		71.3	
5)	56.8	71.0	65.2	60.7	74.5	59.8	75.9	60.6
6)	50.6		62.7	68.6	79.8	61.7		
7)	40.8	____	50.9	____	69.1	83.5	72.1	74.5
$\bar{X}$)	52.4	67.8	52.9	62.9	63.3	60.7	68.2	60.2
Range)	37-66	64-71	33-65	52-78	28-80	45-84	60-76	45-75
Mean RQ	1.29		1.19		0.96		1.03	

slice substrate. Since these animals were sacrificed immediately following their one hour meal, it does not seem likely that there was opportunity to replenish the depleted glycogen reserves incurred during the prior 12 hour fast. However, the RQ of near unity at the beginning of the experiment indicates that predominately carbohydrate was being utilized. As the RQ values of Table I indicate, this supply of carbohydrate was not adequate for the full 120 min. period.

Further information regarding metabolic activity during continued incubation is available from the distribution of the C^{14} label added to the substrate of these tissues. The portion of this tracer recovered in the CO_2, fatty acid and cholesterol fractions is presented as % incorporation* data in Table II.

It is apparent that despite the wide range of values found for the lipid fractions, a fairly constant relationship exists between the four samples from each experiment. Thus it is possible to plot the averages as obtained above, giving a graphic representation of these data. Figure 4 is a semilog plot of these averaged % incorporation data for CO_2, fatty acid and cholesterol, expressed as % incorporation/hr incubation.

CO_2 shows an initial rather sharp decline in the rate of incorporation of label which then stabilizes up to about 90 min. incubation, followed by a further decline as seen in the 2 hr sample. It should be remembered that during this same 2 hr incubation, an 11% decrease was seen in the rate of CO_2 production, compared to a 32% decrease in the incorporation of label into this fraction. Thus, while the rate of CO_2

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*$\%\ \text{incorporation} = \frac{\text{total cpm in tissue fraction}(CO_2, \text{Fatty Acid or Cholesterol})}{\text{total cpm in acetate added to substrate}} \times 100$

Table II
TIME COURSE OF INCUBATION
% INCORPORATION
CO_2, Fatty Acids and Cholesterol

	30 min incubation			60 min incubation		
	CO_2	FA	CHOL	CO_2	FA	CHOL
1)	7.1	0.52	0.67	11.8	0.71	1.23
2)	7.9	3.00	1.72	19.1	5.47	3.95
3)	5.7	0.04	0.47	7.5	0.04	6.70
4)	14.5	0.31	0.85	18.3	0.74	1.21
5)		1.01	0.47		1.10	0.84
$\bar{X}$)	9.2	0.98	0.84	13.8	1.61	1.59
Range	5.7-14.5	0.04-3.00	0.47-1.72	7.5-19.1	0.04-5.47	0.84-3.95
%/Hr	18.4	1.96	16.8	13.8	1.61	1.59

	90 min incubation			120 min incubation		
	CO_2	FA	CHOL	CO_2	FA	CHOL
1)	11.0	0.72	1.75	18.9	0.93	2.13
2)	16.0	5.60	5.27	24.6	6.92	7.33
3)		0.08	1.48	17.4	0.08	1.80
4)	22.7	0.43	1.91	27.0	1.00	2.65
5)	33.4	1.60	0.84	36.6	1.60	1.83
$\bar{X}$)	20.5	1.69	0.84	36.6	1.60	1.83
Range	11.0-33.4	0.08-5.60	0.84-5.27	17.4-36.6	0.08-6.92	1.80-7.33
%/Hr	13.7	1.13	1.50	12.5	1.06	1.58

Figure 4

Time Course of Incubation

Percent of substrate acetate label incorporated into CO_2, fatty acids and cholesterol per hour of incubation. CO_2 values have been divided by a factor of 10. Flasks contained 1 gram of liver slices in 25 ml Krebs-Ringer-Phosphate buffer, pH 7.4 and 1 ml acetate-1-C^{14} (100,000 cpm, 0.253 uM). Incubation was for 2 hours at 37.5°C.

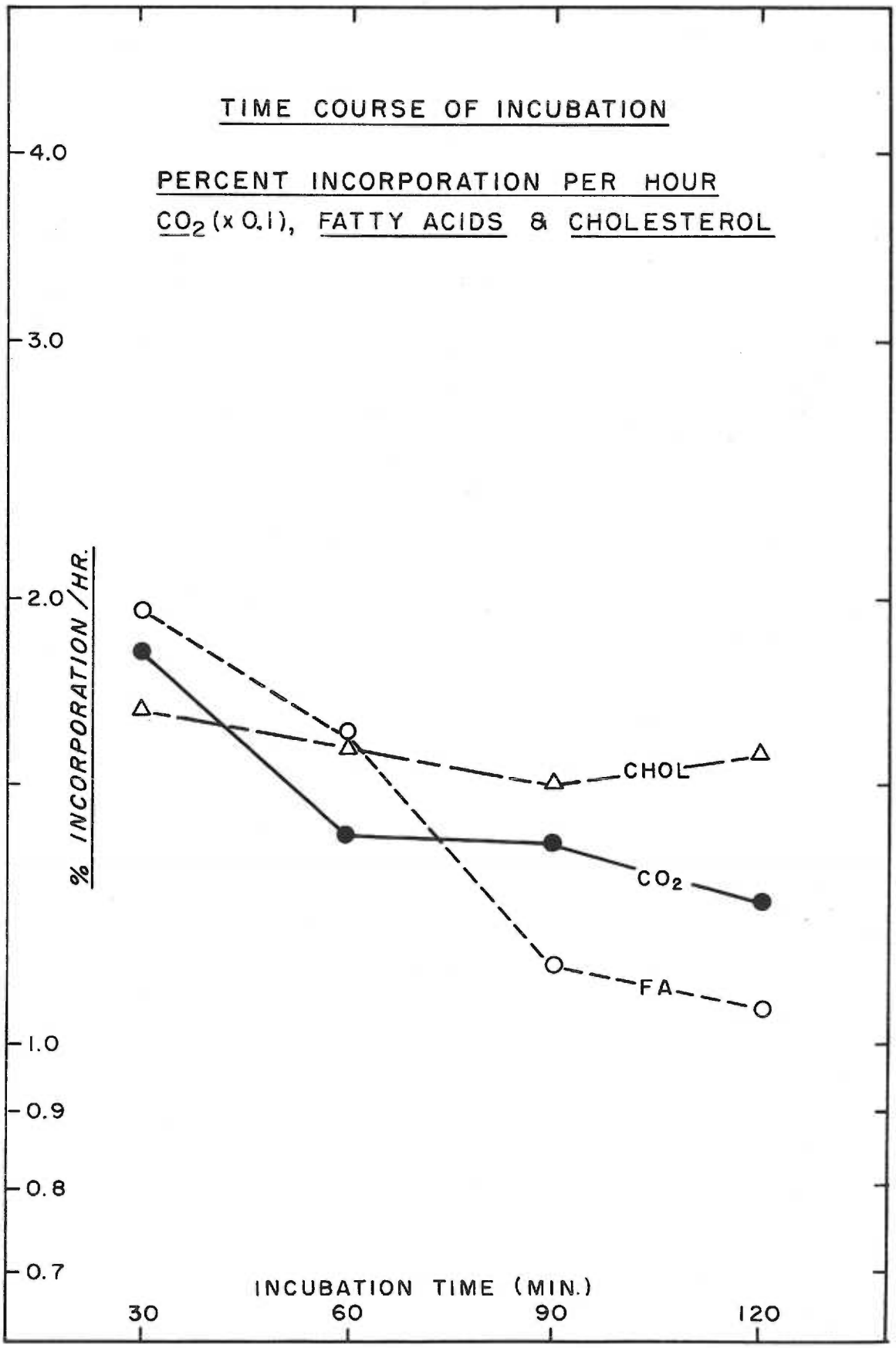
TIME COURSE OF INCUBATION
PERCENT INCORPORATION PER HOUR
CO_2 (x 0.1), FATTY ACIDS & CHOLESTEROL
4.0
3.0
2.0
1.0
0.9
0.8
0.7
% INCORPORATION /HR.
CHOL
CO_2
FA
INCUBATION TIME (MIN.)
30
60
90
120

FIGURE 4

production declines during the incubation period, the incorporation of label into this CO_2 declines at a more rapid rate, resulting in relatively "less labeled" CO_2. This is seen by examination of the CO_2 specific activity* data (Table III). The constant decline in CO_2 specific activity may be interpreted as a diminishing "rate" of oxidation of acetate-1-C^{14} to CO_2 although other interpretations of this change in specific activity will be considered later.

Referenee to Figure 4 shows that the two lipid fractions behave quite differently with regard to the incorporation of label. While the rate of incorporation of label into fatty acid (% incorporation per hour) shows a relatively constant decrease throughout the incubation period (1.96%/hr in the 30 min sample compared to 1.06%/hr in the 2 hr sample), the rate of incorporation of label into cholesterol was fairly constant. This is even more apparent if the magnitude of these changes are considered as % decrease in the rate of incorporation of label. Thus, while the incorporation into fatty acid decreased to 46% of the activity in the initial 30 min sample, the incorporation into cholesterol declined only 6% after two hours of incubation. It will be remembered that the incorporation into CO_2 decreased 32% during this same period.

Again, consideration of specific activity data may be informative since this calculation estimates the activity per unit of metabolite recovered. As seen in Table III, CO_2 specific activity declined 10% during the incubation period as compared to a 32% decrease in the incorporation of label into CO_2. Fatty acids, on the contrary, had a specific activity 2.08 times as great after two hours incubation while

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*specific activity = cpm/uM CO_2 (or cpm per mg. of fatty acid or cholesterol)

Table III
TIME COURSE OF INCUBATION
SPECIFIC ACTIVITY
CO_2, Fatty Acids and Cholesterol

	30 Min Incubation			60 Min Incubation		
	CO_2	FA	CHOL	CO_2	FA	CHOL
1)	215	17.5	303	201	26.0	787
2)	223	104	169	236	180	1830
3)		1.12	191		1.33	248
4)	342	23.8	336	279	25.6	582
5)		39.1	231		47.7	456
$\bar{X}$)	260	37.1	246	239	56.1	781
Range	215–342	1.12–104	169–336	201–279	1.33–180	248–1830

	90 Min Incubation			120 Min Incubation		
	CO_2	FA	CHOL	CO_2	FA	CHOL
1)	158	28.6	759	201	33.4	1013
2)	202	192	2568	196	241	3376
3)		2.21	631		2.54	689
4)	241	30.3	991	210	41.0	1291
5)	242	65.4	362	235	67.9	983
$\bar{X}$)	200	63.7	1062	202	77.2	1470
Range	158–242	2.21–192	362–2568	196–235	2.54–241	689–3376

the rate of incorporation of label into fatty acid decreased 46%. This is even more striking for cholesterol where a 6% decrease in the rate of incorporation into this lipid is accompanied by a specific activity 5.98 times as great at the end of incubation as was found in the 30 min sample.

This relationship of specific activity in the three metabolic compartments being studied is expressed graphically in Figure 5. From this it might be reasoned that while the rate at which C^{14} label is being incorporated into CO_2, fatty acid and cholesterol decreases during the incubation period (Figure 4), the amount of this C^{14} label per unit of metabolite (Figure 5) decreases only in CO_2 but increases 2-fold in fatty acid and 6-fold in cholesterol. For CO_2, this is partially explainable by the fact that the amount of CO_2 being produced decreases during the incubation period, thus a smaller quantity of CO_2 contains relatively the same concentration of label (actually 10% less). On the other hand, the actual amount of the two lipids remains quite constant during the incubation period (see Table IV). Thus, while the rate at which label is incorporated into these lipids declines (more so for fatty acid than cholesterol), the actual density or concentration of this label increases due to the lack of measurable increase in the mass of the lipids. Whether this increase in concentration is due to a large quantity of label present in a few newly synthesized molecules of lipid or is due to a relatively uniform labeling of all molecules cannot be answered on the basis of the present data.

The decline in rate of label incorporation, without measurable change in lipid compartment size may be explained on the basis of a shift in metabolic equilibrium as shown below. At all times, in the

Figure 5

Time Course of Incubation

Specific activity of CO_2, fatty acids and cholesterol during the first two hours of incubation. Specific activity reported as cpm per mg. lipid or per uM CO_2. Fatty acid values have been multiplied by a factor of 10. Flasks contained 1 gram of liver slices in 25 ml Krebs-Ringer-Phosphate buffer, pH 7.4 and 1 ml acetate-1-C^{14} (100,000 cpm, 0.253 uM). Incubation was for 2 hours at 37.5°C.

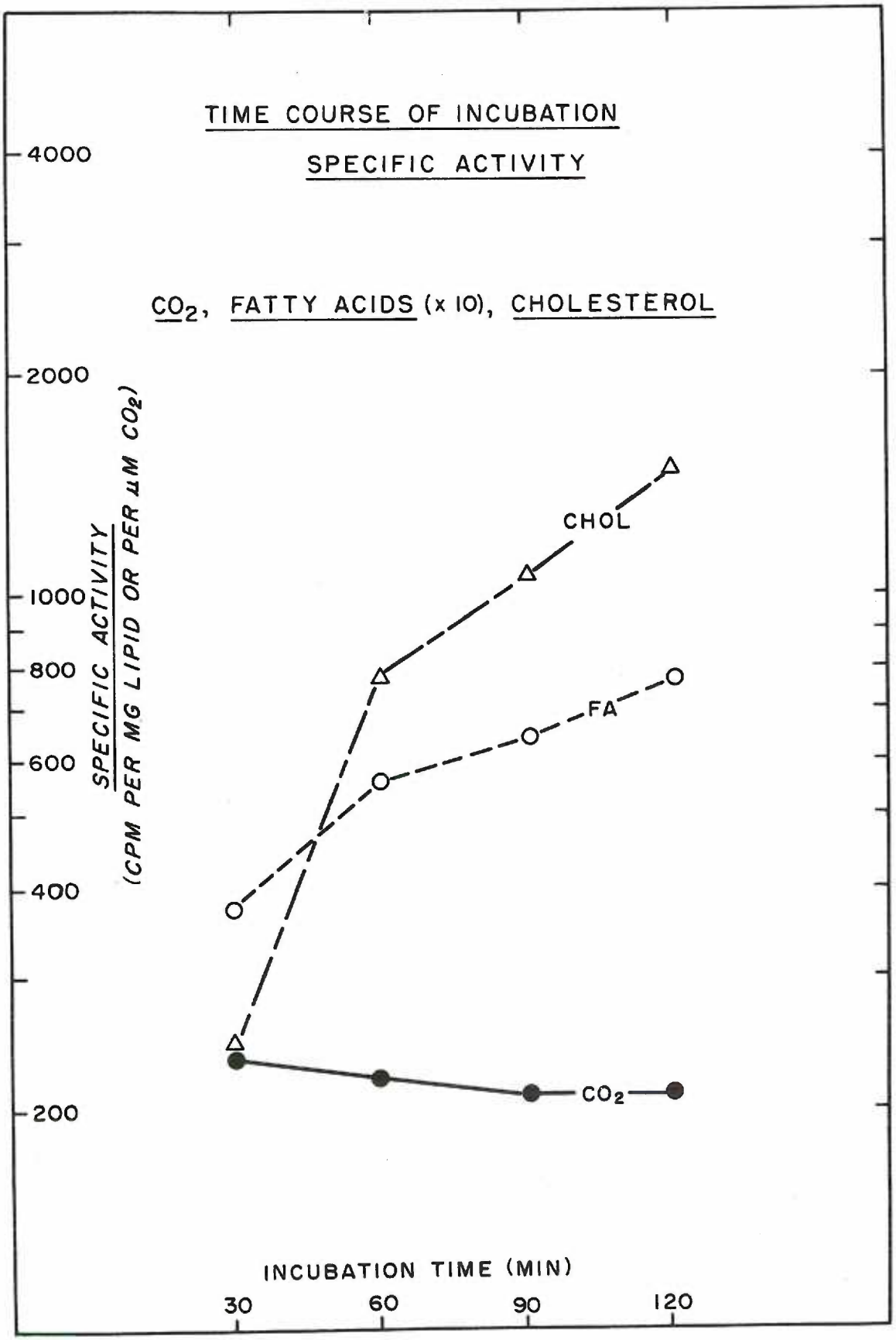
TIME COURSE OF INCUBATION
SPECIFIC ACTIVITY
CO2, FATTY ACIDS (x 10), CHOLESTEROL
4000
2000
1000
800
600
400
200
SPECIFIC ACTIVITY
(CPM PER MG LIPID OR PER µM CO2)
CHOL
FA
CO2
INCUBATION TIME (MIN)
30
60
90
120

FIGURE 5

TABLE IV
TIME COURSE OF INCUBATION
Recovery of Fatty Acids and Cholesterol
(Mg. Lipid/Gm. Tissue)

FATTY ACIDS

	Minutes of Incubation			
	30 Min	60 Min	90 Min	120 Min
1)	16.2		17.4	
2)	31.6	26.1	24.5	27.2
3)	27.0	29.6	29.9	27.8
4)	34.0	43.5	32.0	31.6
5)	12.5	28.6	14.1	24.0
6)	23.8	22.1	25.9	
7)	24.1	22.5	22.3	22.7
$\bar{X}$)	24.2	28.7	23.7	26.7

CHOLESTEROL

	Minutes of Incubation			
	30 Min	60 Min	90 Min	120 Min
1)	2.30		2.40	
2)	2.30	2.10	2.20	2.00
3)	1.77	1.97	2.00	1.97
4)	2.46	2.91	2.34	2.61
5)	2.43	2.05	1.93	2.01
6)	1.21	1.35	1.53	
7)	1.86	1.85	1.81	1.78
$\bar{X}$)	2.05	2.02	2.03	2.07

slice as well as in the intact rat, it is assumed that the following equilibrium exists:

$$\text{Carbohydrate} \longrightarrow \text{Active Acetate} \underset{B}{\overset{A}{\rightleftharpoons}} \text{Fatty Acids.}$$

During the early part of the experiment (30 min) it is likely that dietary carbohydrate supplied the greater part of the energy requirements (i.e., the RQ was about 1.0). At this time, reaction B would be expected to predominate, that is, the equilibrium would shift toward fat storage. Predominance of synthesis over degradation would favor the drawing of appreciable amounts of labeled acetate-1-C^{14} substrate into the lipid compartment. At later times, as carbohydrate supplies decrease, the equilibrium is shifted to the left (i.e., reaction A predominates) and less C^{14} label is "fixed" in the lipid compartment per unit of time.

Another factor that must be considered at this point is the nature of, and the specific activity of, the acetyl CoA precursor of all three metabolic compartments that were studied. Should an influx of unlabeled acetate (endogenously formed?) cause the specific activity of the acetylating precorsor to decrease, a decreased specific activity and a decreased % incorporation and a decreased rate of incorporation might well result. The information available from the CO_2 and fatty acid data indicates that this phenomena might explain the changes seen in these fractions. The data of the cholesterol fractions indicates that sterol C^{14} activity, as specific activity, % incorporation, and % incorporation per hour was not subject to a gross dilution of the specific activity of the acetylating precursor. If a common acetyl CoA source furnishes acetate carbon for the reactions followed in this study, we may conclude

that we have indeed followed changes in the rate of CO_2 and fatty acid formation in the studies described in this thesis.

A final possibility has however not been excluded. Dilution of the acetate "pool" by exogenous acetate will not necessarily cause lowered label incorporation, for if reaction B above is stimulated by an increase in the concentration of acetate, then the dilution effect might be offset by an increased rate of synthesis--resulting in "normal" label incorporation. To postulate a situation such as this, it is again necessary to say that the same acetate "pool" is not common to all the three systems studied, for the nulifying effects postulated for the cholesterol system should also play a role in the CO_2 fatty acid systems.

Effect of Fasting on Time Course of Incubation

In previous discussion it was pointed out that the animals used in these experiments were probably not optimal with regard to nutrition since they were sacrificed too soon postprandially for any significant energy reserves to be laid down in the liver. To test this assumption it was necessary to repeat this study using fasted animals. For this purpose, three 12 hr fasted rats were used, along with three 60 min fed rats to act as a control group. Flasks were removed from the Warburg bath at 1, 2 and 3 hours incubation. The results of these experiments are presented in Tables V, VI and VII.

Results

O_2 utilization and CO_2 production are presented in Table V. From these data, it is apparent that in both fed and fasted slices, O_2 utilizations increased slightly during incubation while CO_2 production decreases, resulting in a fall in RQ values from unity to 0.70 during

Table V
TIME COURSE OF INCUBATION
O_2 and CO_2
uM/Gm/Hr

Fed (1 Hr)

	1 Hr Incubation		2 Hr Incubation		3 Hr Incubation	
	O_2	CO_2	O_2	CO_2	O_2	CO_2
1)	71.9	57.3	76.6	53.6	73.9	49.9
2)	70.9	51.9				
3)	67.8	58.3	66.3	49.8	69.3	49.2
4)	66.0	53.4				
5)	61.2	57.3	61.3	47.9	63.9	47.3
6)	58.4	63.6				
$\bar{X}$)	66.0	57.0	68.1	50.4	69.0	48.8
Range	58-72	52-64	61-77	48-54	64-74	47-50
RQ	0.86		0.74		0.71	

Fasted (12 Hr)

	1 Hr Incubation		2 Hr Incubation		3 Hr Incubation	
	O_2	CO_2	O_2	CO_2	O_2	CO_2
1)	58.5	48.8	63.4	40.0	64.7	43.9
2)	59.7	47.7				
3)	59.2	68.9	64.7	57.3	67.7	49.1
4)	63.8	75.5				
5)	64.6	57.5	65.6	50.2	69.2	52.9
6)	65.4	75.2				
$\bar{X}$)	61.9	62.3	64.6	49.2	67.2	48.6
Range	59-65	48-76	63-66	40-57	65-69	44-53
RQ	1.01		0.76		0.72	

Table VI
TIME COURSE OF INCUBATION
% INCORPORATION
CO_2, Fatty Acids, and Cholesterol

Fed (1 Hr)

	1 Hr Incubation			2 Hr Incubation			3 Hr Incubation		
	CO_2	FA	CHOL	CO_2	FA	CHOL	CO_2	FA	CHOL
1)	12.6	0.33	1.74	21.2	0.40	2.86	27.4	0.42	3.60
2)	11.2	0.35	1.97						
3)	12.4	0.95	2.08	19.5	0.98	4.05	27.7	1.01	4.92
4)	11.8	0.94	2.10						
5)	12.0	0.76	1.22	21.2	0.97	2.28	26.8	0.78	2.72
6)	11.5	0.69	1.21						
$\bar{X}$)	11.9	0.67	1.72	20.6	0.78	3.06	27.3	0.74	3.75
Range	11-13	0.3-1.0	1.2-2.1	20-21	0.4-1.0	2.3-4.1	27-28	0.4-1.0	2.7-4.9
%/Hr	11.9	0.67	1.72	10.3	0.39	1.53	9.1	0.25	1.25

Fasted (12 Hr)

	1 Hr Incubation			2 Hr Incubation			3 Hr Incubation		
	CO_2	FA	CHOL	CO_2	FA	CHOL	CO_2	FA	CHOL
1)	9.02	0.98	1.56	15.6	0.88	2.43	23.8	0.99	2.95
2)	8.27	1.09	1.58						
3)	16.2	0.55	1.17	22.1	0.64	1.84	24.4	0.69	2.11
4)	16.6	0.46	1.10						
5)	9.53	0.15	0.59	18.1	0.22	0.73	25.0	0.18	0.83
6)	10.9	0.22	0.48						
$\bar{X}$)	11.8	0.58	1.08	18.6	0.58	1.67	24.4	0.62	1.96
Range	8-17	0.2-1.1	0.5-1.6	16-22	0.2-0.9	0.7-2.4	24-25	0.2-1.0	0.8-3.0
%/Hr	11.8	0.58	1.08	9.3	0.29	0.84	8.1	0.21	0.65

Table VII
TIME COURSE OF INCUBATION
SPECIFIC ACTIVITY
CO_2, Fatty Acids and Cholesterol

Fed (1 Hr)

	1 Hr Incubation			2 Hr Incubation			3 Hr Incubation		
	CO_2	FA	CHOL	CO_2	FA	CHOL	CO_2	FA	CHOL
1)	207	12.7	754	199	15.2	1269	184	18.1	1651
2)	212	10.2	925						
3)	201	35.6	1067	188	32.2	2048	179	33.9	2764
4)	205	26.6	1024						
5)	213	32.5	630	212	31.4	1200	190	31.0	1337
6)	174	27.8	603						
$\bar{X}$)	202	24.2	834	199	26.3	1505	184	27.7	1917
Range	174–213	10.2–35.6	603–1067	188–212	15.2–32.2	1200–2048	179–190	18.1–33.9	1337–2764

Fasted (12 Hr)

	1 Hr Incubation			2 Hr Incubation			3 Hr Incubation		
	CO_2	FA	CHOL	CO_2	FA	CHOL	CO_2	FA	CHOL
1)	178	33.8	788	190	30.5	1138	177	36.0	1473
2)	168	36.8	788						
3)	231	20.1	568	191	24.0	918	165	28.0	1053
4)	216	18.6	526						
5)	164	8.32	219	177	8.75	372	156	10.4	503
6)	143	7.61	231						
$\bar{X}$)	183	20.9	520	186	21.1	809	166	24.8	1010
Range	143–231	7.61–36.8	219–788	177–191	8.75–30.5	372–1138	156–177	10.4–36.0	503–1473

incubation. Considered separately, O_2 utilization increased 5% in the fed animals and 9% in the fasted animals while CO_2 production decreased 14% in the fed and 22% in the fasted animals. Thus it appears that carbohydrate energy stores are depleted slightly more rapidly in the fasted animal than in the fed animal, as would be anticipated.

The distribution of the C^{14} label is summarized for these experiments in Table VI, while Figure 6 presents the data graphically. These data indicate that the standard one hour feeding period is ample time for the ingested food to influence hepatic metabolism. In every case, the incorporation of label into CO_2, fatty acid and cholesterol was greater in the fed than in the fasted animals showing that the primarily catabolic processes of the fasted animal were shifting to more synthetic activity after the feeding period. This increased rate of incorporation was greatest for cholesterol (averaging 78% higher) and least for CO_2 (8%). It was unexpected to find that cholesterol should show the greatest response to feeding since it usually shows a less dramatic response to nutritional changes than does fatty acid. The small change seen for CO_2 is compatable with more comprehensive data on the influence of fasting to be presented later.

The factor of primary concern at this point is the influence of prior nutritional status on metabolic activity during incubation of the slices. The data appear to indicate that there is no great difference in the two preparations as far as lipogenic activity is concerned up to at least three hours of incubation since the slopes at which the rate of incorporation of label is decreasing appear to be approximately the same in the fed and fasted animal if the initial difference in incorporation is disregarded.

Figure 6

Time Course of Incubation

A comparison of the percent of substrate acetate label incorporated into CO_2, fatty acids and cholesterol, at various incubation times, by slices obtained from the livers of fed and 12 hr. fasted rats. Solid lines represent slices from fed rats and broken lines represent slices from fasted rats. CO_2 values have been divided by a factor of 10. Flasks contained 1 gram of liver slices in 25 ml Krebs-Ringer-Phosphate buffer, pH 7.4 and 1 ml acetate-1-C^{14} (100,000 cpm, 0.253 uM). Incubation was for 2 hours at 37.5°C.

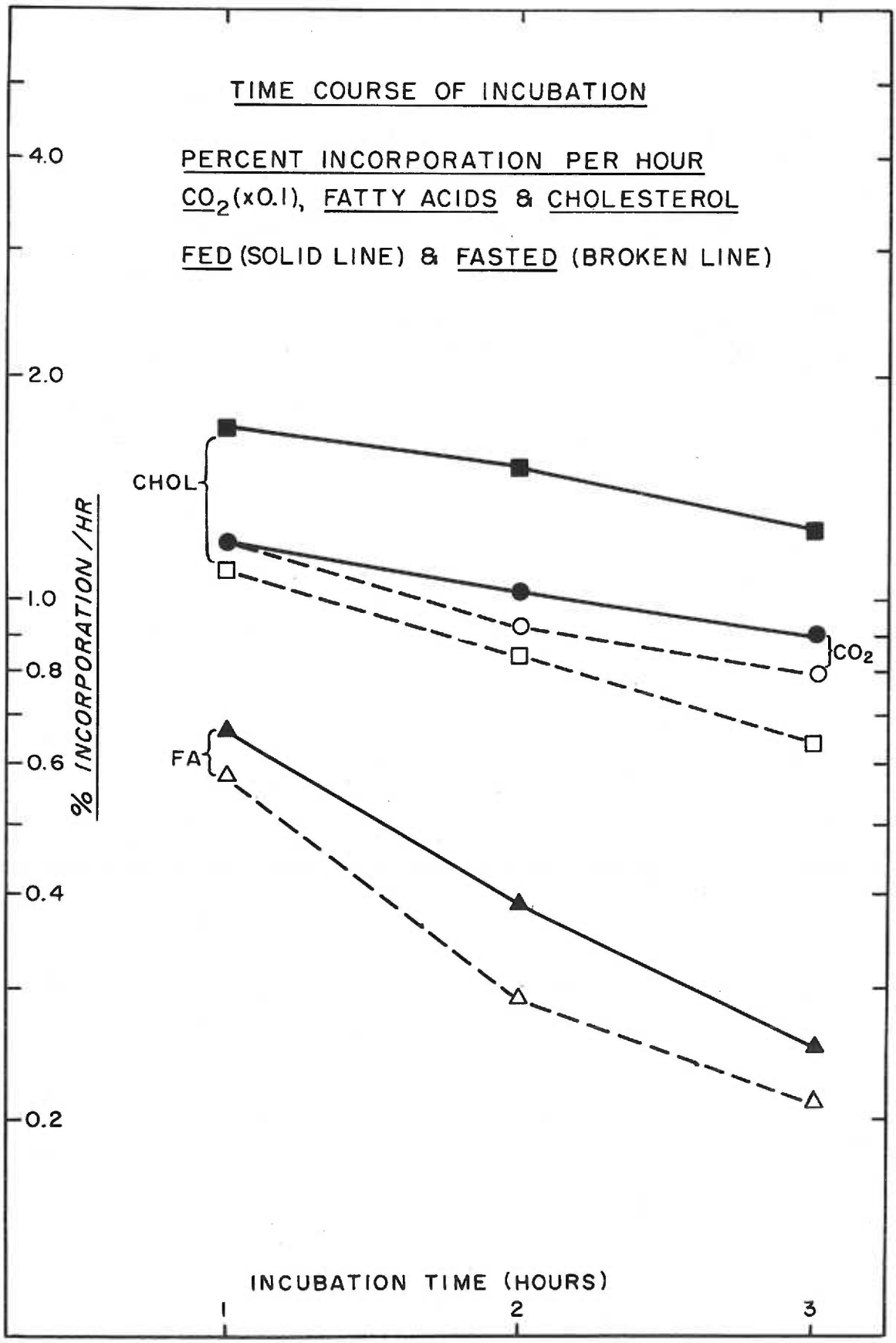
TIME COURSE OF INCUBATION
PERCENT INCORPORATION PER HOUR
CO_2(x0.1), FATTY ACIDS & CHOLESTEROL
FED (SOLID LINE) & FASTED (BROKEN LINE)
4.0
2.0
1.0
0.8
0.6
0.4
0.2
% INCORPORATION /HR
CHOL
CO_2
FA
INCUBATION TIME (HOURS)
1
2
3

FIGURE 6

Table VII and Figure 7 shows essentially the same changes in specific activity as were presented previously (Figure 6). Again, although the absolute values differ, the slopes for the fed and fasted animals remain similar showing that although feeding influences the absolute amount of label incorporated into CO_2, fatty acid and cholesterol of surviving liver slices, it has little influence on the gradual decrease in the rate at which the processes are going on during the course of incubation.

Summary

In summary, liver slices from fed and fasted rats were incubated for various periods of time from 30 min to three hours in the presence of acetate-1-C^{14} tracer. During the course of incubation, there is a gradual decrease in the rate at which the label is incorporated into CO_2, fatty acid and cholesterol; this decrease being greatest for fatty acid (63% less), and less for CO_2 (27%) and cholesterol (33%). This decrease is, however, largely independent of the previous nutritional status of the animal, the slopes being similar in both the fed and fasted animals. That there was an actual difference in the nutritional status of the two series of animals is shown by the actual % incorporation data for any given period of incubation. Thus, "fed slices" incorporated 23% more label into fatty acid than did "fasted slices" and 78% more label into cholesterol.

Time Course of Fasting

Introduction

As pointed out in the introductory chapter, one of the principle reasons for these in vitro studies is the extension and confirmation of

Figure 7

Time Course of Incubation

A comparison of the specific activities of CO_2, fatty acids and cholesterol, during incubation with acetate-1-C^{14}, using slices from either fed (solid line) rats or 12 hour fasted rats (broken line) are used. Cholesterol values have been divided by a factor of 10. Flasks contained 1 gram of liver slices in 25 ml Krebs-Ringer-Phosphate buffer, pH 7.4 and 1 ml acetate-1-C^{14} (100,000 cpm, 0.253 uM). Incubation was for 2 hours at 37.5°C.

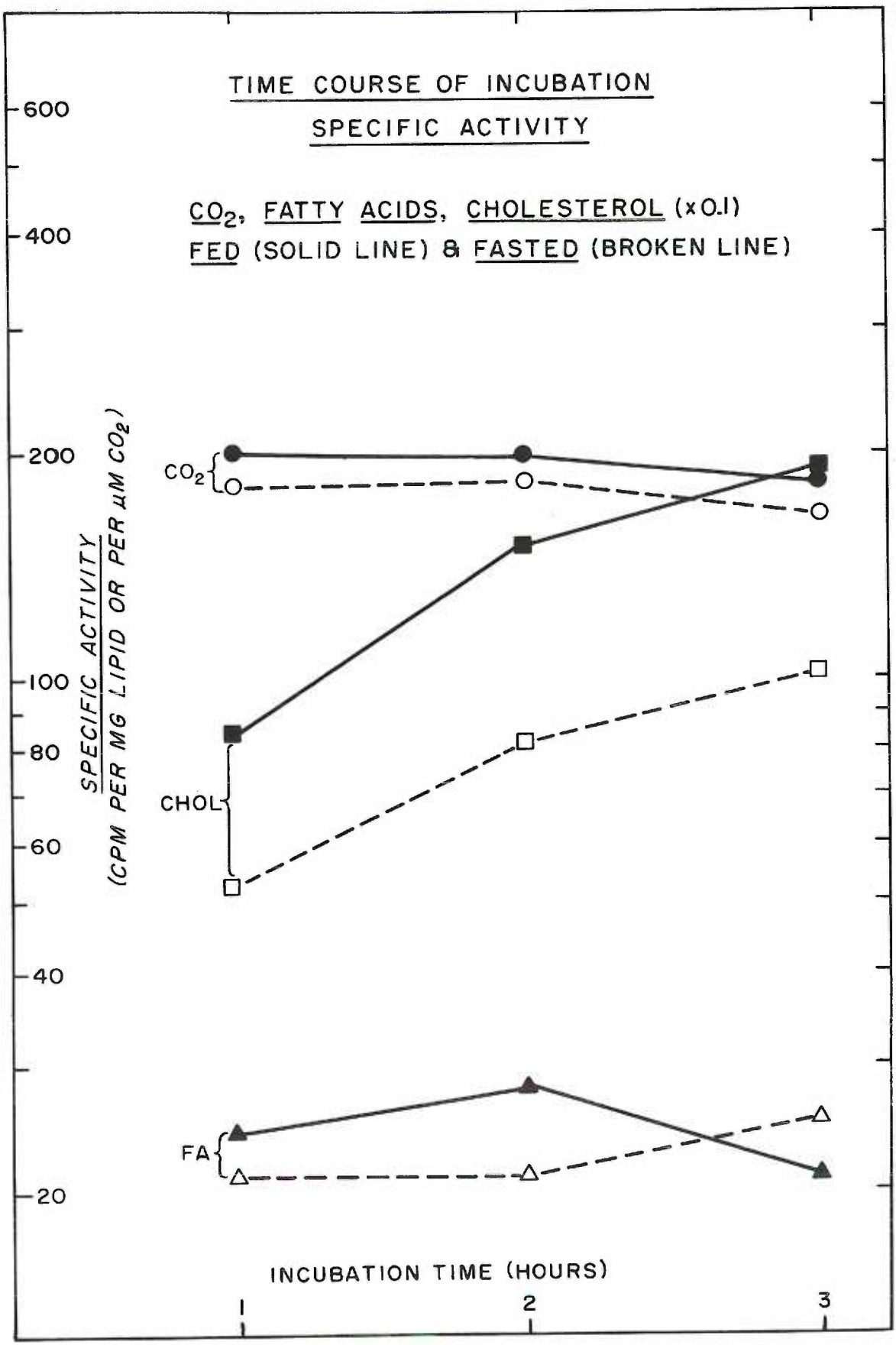
TIME COURSE OF INCUBATION
SPECIFIC ACTIVITY
CO2, FATTY ACIDS, CHOLESTEROL (x0.1)
FED (SOLID LINE) & FASTED (BROKEN LINE)
SPECIFIC ACTIVITY
(CPM PER MG LIPID OR PER µM CO2)
600
400
200
100
80
60
40
20
CO2
CHOL
FA
INCUBATION TIME (HOURS)
1
2
3

FIGURE 7

intact animal studies already reported from this laboratory. One of the phases of our intact animal studies where in vitro correlation is desirable is the problem of the influence of fasting on hepatic lipogenesis. Certain aspects of this problem have already been alluded to in the previous section on the time course of incubation.

In intact animal studies, Cockburn[60] has shown that hepatic fatty acid uptake of acetate-1-C^{14} (i.e., synthesis) increases rapidly following the refeeding of 12 hr fasted rats. Within 45 min from the time the animals are allowed access to food, 4.5 times as much of the intraperitoneally injected acetate label is found in the liver fatty acid fraction as is present following a similar injection in a 12 hr fasted animal. This increased incorporation continues up to about the third hour of fasting where the amount of label fixed in the fatty acids is about 6.5 times the 12 hr fasted value. A rapid decline then occurs and 2 hrs later (5 hr fasted rat) the incorporation into fatty acid is only about twice the 12 hr fasted level and by the eighth hour of fasting, the incorporation of label has returned nearly to the 12 hr base line.

The in vivo labeling of hepatic cholesterol at these same time intervals following the refeeding of a 12 hr fasted animal is quite different from the responses observed for fatty acid. Unlike fatty acid, there is a slightly diminished incorporation of label into cholesterol following the feeding period, so that at the end of this 60 min period the incorporation of label into cholesterol is nearly 20% less than that seen in the 12 hr fasted animal. This may be attributable to the influx of non-labeled dietary cholesterol with consequent inhibition of hepatic cholesterol synthesis, but this point has not been established with

certainty. A maximum increase in the "fixation" of label into cholesterol occurs about one hour postprandially and amounts to about 15% more than in the fasted animal. This is in contrast to the 6.5 fold increase seen in fatty acid labeling. Following the increased cholesterol synthesis seen in the one hr fasted rat, there is a decline in acetate incorporation so that at the third hr of fasting only 90% of the 12 hr incorporation is seen and by the fifth hour, only slightly more than half of the 12 hr incorporation is found. A steadily increasing incorporation then occurs with continued fasting which by the eighth hour is 25% below the 12 hr value.

In vivo CO_2 data shows that there is essentially no change in the amount of the added acetate label which is recovered as $C^{14}O_2$ at the various intervals following refeeding. A discussion of these data as well as the responses reported for fatty acid and cholesterol in vivo will be discussed at the end of this section.

Methods

In order to study this problem in vitro, a total of 36 rats were prepared as before and sacrificed at intervals during and following the standard one hr feeding period. Liver slices were prepared as previously described and 1 gm. of slices/flask was incubated for 1 hr at 37.5°C in Krebs-Ringer-Phosphate medium containing 100,000 cpm Na Acetate-1-C^{14} (0.253 uM acetate/26 ml substrate volume).

Results and Discussion

Respiration—Oxygen utilization and CO_2 production data are reported in Table VIII. These data are reported as uM of O_2 utilized or CO_2 produced per gram wet weight of slices per hour of incubation.

Table VIII

TIME COURSE OF FASTING

GAS EXCHANGE

uM/Gm. Tissue/Hr

FAST	O_2		CO_2		RQ
	uM	SE*	uM	SE*	
Zero**	63.6	2.8	53.9	2.3	0.85
1 hr	58.1	0.6	52.7	2.9	0.91
2 hr	57.0	1.7	57.9	2.5	1.00
4 hr	56.2	1.5	51.7	2.3	0.92
6 hr	58.0	2.3	58.6	1.4	1.00
12 hr	62.4	1.3	61.3	3.6	0.98

* SE = standard error of mean

**Rats killed immediately following 60 min feeding period

Both O_2 utilization and CO_2 production showed a slight decrease from maximum values in the non-fasted animals (zero hour) to a minimum at the fourth hour following the 60 min feeding period. Although CO_2 production appears to drop slightly more than O_2 utilization during this period this is more an apparent difference than a real one since the RQ remains fairly constant between 0.90 and 1.00 throughout the feeding-fasting period. If the standard errors of these means are considered, it is seen that the differences observed are not incompatable with expected sampling errors. Thus, the maximum change seen in O_2 utilization is about 7 uM while the 95% confidence interval of the means with an average standard error of 1.7 uM would be nearly 5 uM. Application of the "t-test" for the significance of the difference between means further verifies the probability that if real differences are present, they are small. An additional factor that limits the interpretation of the RQ data is the fact that the slices were subjected to the hour of "fasting" involved in their preparation, in addition to which the experiment itself is conducted over an hour period, an additional hour of deprivation of metabolites ordinarily supplied by the circulation.

% incorporation data for the three fractions (CO_2, fatty acids and cholesterol) are presented in Table IX while Table X summarizes the specific activity data.

In these tables, fasting is represented as the time interval following the standard 60 min feeding period (i.e., zero fast means that the animal was sacrificed immediately following the feeding period, etc.). For each of the three fractions, data are presented as % of the

Table IX
TIME COURSE OF FASTING
% INCORPORATION
CO_2, Fatty Acids and Cholesterol

FAST	CO_2			Fatty Acids			Cholesterol		
	%*	Mean	SE**	%*	Mean	SE**	%*	Mean	SE**
Zero	11.9	11.4	0.28	0.34	0.66	0.13	1.86	1.79	0.15
	12.1			0.95			2.09		
	11.8			0.73			1.22		
	10.2			0.60			2.00		
	11.0						1.80		
1 hr	8.12	10.1	0.79	0.63	0.92	0.13	0.80	1.68	0.40
	9.45			1.01			2.31		
	11.4			1.23			1.23		
	11.3			0.81			2.39		
2 hr	13.2	11.7	0.78	4.00	3.98	0.41	4.49	3.72	0.61
	12.8			4.96			3.42		
	9.82			2.96			2.14		
	11.1			4.00			4.82		
4 hr	8.11	9.77	0.53	2.01	2.99	0.53	1.70	2.52	0.32
	10.3			2.47			1.90		
	7.54			1.00			1.53		
	9.55			5.05			2.55		
	8.78			1.57			2.91		
	10.1			6.48			1.56		
	8.80			2.16			2.65		
	8.25			6.02			4.18		
	8.94			3.24			2.50		
	13.9			1.76			1.77		
	10.7			2.50			1.95		
	12.3			1.61			5.02		
6 hr	12.3	11.8	0.37	2.54	1.73	0.45	1.35	0.91	0.16
	12.2			2.41			0.59		
	10.7			1.27			0.77		
	11.9			0.69			0.92		
12 hr	8.65	10.8	1.22	1.04	0.47	0.13	1.57	1.35	0.28
	16.4			0.51			1.14		
	10.2			0.19			0.54		
	11.1			0.47			2.53		
	7.96			0.43			1.45		
	10.3			0.17			0.87		

* Each number represents the mean % incorporation of 2 or more samples from a single animal.
** SE = standard error of mean.

Table X
TIME COURSE OF FASTING
SPECIFIC ACTIVITY
CO_2, Fatty Acids and Cholesterol

FAST	CO_2			Fatty Acids			Cholesterol		
	SA*	Mean	SE**	SA*	Mean	SE**	SA*	Mean	SE**
Zero	210	200	3.4	11.5	24.3	4.5	840	858	77
	203			3.31			1046		
	194			30.2			617		
	203			24.4			998		
	191						791		
1 hr	151	185	19	25.0	37.2	5.5	380	816	210
	167			42.6			1214		
	239			49.6			535		
	184			31.6			1136		
2 hr	202	199	8.0	149	147	13	2182	1910	278
	220			175			1939		
	187			111			1125		
	186			152			2393		
4 hr	144	186	12	66.7	108	17	870	1235	166
	169			86.6			948		
	131			37.2			765		
	211			207			1342		
	184			61.4			1636		
	155			205			561		
	151			153			1175		
	153			112			2090		
	225			83.3			1280		
	263			102			706		
	228			70.3			1000		
	214						2450		
6 hr	186	195	3.9	109	73.8	22	577	418	59
	205			113			395		
	193			47.4			383		
	196			25.7			418		
12 hr	173	171	12	35.3	17.4	4.2	788	644	127
	224			19.4			547		
	154			7.97			225		
	173			18.6			1076		
	147			17.1			825		
	153			602			401		

* Each specific activity represents the mean of 2 or more samples from a single animal.
** SE = standard error of mean.

substrate label incorporated into the metabolic product (Table IX) and as activity (cpm) recovered per uM of CO_2 or per mg. of fatty acid or cholesterol (Table X). Each value in the % incorporation or specific activity columns represents the mean of two or more flasks of liver slices from a single animal.

Since little or no change is found in the amount of CO_2, fatty acid or cholesterol recovered from a gram of slices at the various time intervals of fasting, the specific activity calculations add little to an analysis of the results. For this reason, this discussion will deal primarily with the % incorporation data.

Fatty Acid—The changes in fatty acid labeling at the various intervals following refeeding are shown in the second column of Table IX. These mean % incorporation figures may be plotted graphically either as % incorporation per se, or as changes with respect to the mean value obtained for the 12 hr fasted animal. The justification for this computation is the assumption that the 12 hr fasted animal represents the maximum duration of fasting prior to the 60 min refeeding period as established during the trained-feeding regime to which all animals were subjected. This twelfth hour of fasting may then be considered the base line from which the alterations induced by refeeding and subsequent fasting will occur and therefore the base line to which these alterations may be compared. By this calculation, increases in the incorporation of acetate-1-C^{14} label into either fatty acids or cholesterol will be represented by the number of times greater than the 12 hr fasted value which these increases represent, and conversely decreases will be represented by the proportion of the 12 hr fasted value which these decreased

values represent. This calculation has been employed in arriving at the "normalized" % incorporation data plotted in Figure 8.

These graphs indicate that, as previously discussed, the fraction of the substrate label "fixed" in CO_2 does not change significantly at the various intervals after feeding. This CO_2 data is therefore represented by a horizontal line extending along the abscissa at 1.0.

Fatty acids show a marked increase in the incorporation of acetate label in response to feeding which may most likely be interpreted as a marked increase in the synthesis of this lipid. This rise is gradual during the feeding period and the first hour postprandial, the incorporation increase being only about 2 fold at the end of 1 hr of fasting. There follows an abrupt rise in fatty acid activity which at the end of the second hour of fasting has reached a maximum value nearly $8\frac{1}{2}$ times the 12 hr fasted value. (This peak represents an actual % incorporation of nearly 4%). This two hour peak is followed by a constant decrease in incorporation up to at least the sixth hour of fasting. Synthetic activity between 6 and 12 hrs of fasting was studied in only one experiment which was considered technically unsatisfactory and is not reported here. Further investigation of this 6 hr period of fasting is necessary to establish the slope of the % incorporation curve subsequent to the 6 hr fast period.

Cholesterol—The curve for cholesterol (Figure 8) shows the same gradual rise during the feeding period and first hour of fasting as was seen for fatty acid. This initial rise is however more gradual, being only about 25% above the 12 hr fast level one hour after feeding whereas fatty acid incorporation was nearly twice the fasting value at the same

Figure 8

Time Course of Fasting

Labeling response in CO_2, fatty acids and cholesterol with liver slices obtained from fed or 1, 2, 4, 6 or 12 hour fasted rats. Labeling response represents the response of the system in question to either feeding or fasting when compared with slices obtained from 12 hour fasted rats. Horizontal broken line represents CO_2 which is uninfluenced by refeeding and fasting, and therefore this line also represents the base line for comparison with the changes observed in the two lipids. Lipid labeling response = % incorporation at indicated fast period/% incorporation in the 12 hour fasted preparation. Flasks contained 1 gram of liver slices in 25 ml Krebs-Ringer-Phosphate buffer, pH 7.4 and 1 ml acetate-1-C^{14} (100,000 cpm, 0.253 uM). Incubation was for 2 hours at 37.5°C.

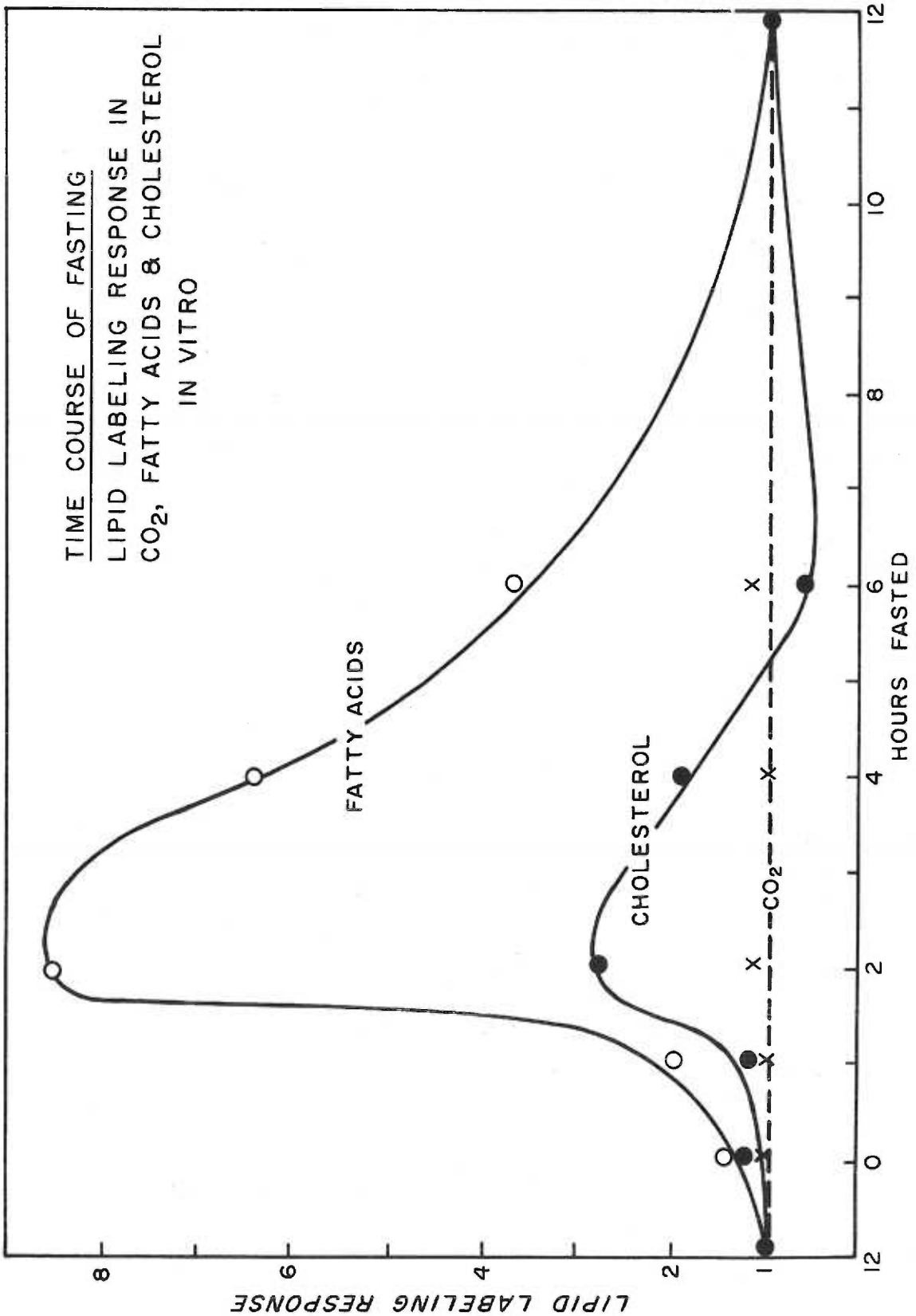
TIME COURSE OF FASTING
LIPID LABELING RESPONSE IN
CO_2, FATTY ACIDS & CHOLESTEROL
IN VITRO
FATTY ACIDS
CHOLESTEROL
CO_2
LIPID LABELING RESPONSE
HOURS FASTED
12
10
8
6
4
2
0
12
1
2
4
6
8

FIGURE 8

interval. Cholesterol also resembles fatty acid in that a maximum increase in the synthesis of this sterol is found 2 hrs after feeding with a subsequent decline in synthetic activity. The maximum rise in label incorporation into cholesterol is however less than three times the 12 hr incorporation value as compared to an $8\frac{1}{2}$ fold increase found for fatty acid.

In addition to the lower peak seen for cholesterol, it will be seen that the incorporation of label into this fraction subsequently falls to levels significantly below the 12 hr fast level during the sixth hour after feeding. Again, the remainder of this curve requires further investigation to establish the slope during the sixth to twelfth hour of fasting.

It is interesting to note the amount of dispersion of the observed % incorporation figures for the three fractions reported. This dispersion, as indicated by the standard error calculations reported in Table IX, may be expressed in terms of the coefficient of dispersion $\frac{\text{standard error}}{\text{mean}}$ making a direct comparison between the various standard errors possible. It was found that the standard error for CO_2 averaged about 6% of the reported means while it was about 16% for cholesterol and nearly 19% for fatty acids. Thus the least dispersion was found for CO_2 and the greatest for fatty acid, with cholesterol being only slightly less. This is consistent with repeated observations on both in vivo and in vitro preparations in this laboratory which indicate that fatty acid labeling is relatively labile while cholesterol labeling is significantly less labile and CO_2 labeling is quite stable. This has been observed under many different experimental conditions.

Summary of In Vitro Data

In summary, the following have been established for liver slices obtained from fed and fasted rats incubated with "tracer concentrations" of acetate-1-C^{14} label:

1). No change in O_2 utilization, CO_2 production or labeling of CO_2 following refeeding and subsequent fasting of 12 hr fasted animals.

2). Nearly three times as much incorporation of label into cholesterol as into fatty acid in the 12 hr fasted animal.

3). 37 times as much incorporation per mg. of cholesterol as per mg. of fatty acid in the 12 hour fasted animal.

4). A gradually increasing incorporation of label into fatty acid and cholesterol during the 60 min feeding period and the first hour following feeding, representing a 25% increase for cholesterol and a 96% increase for fatty acid over the 12 hr fasted level.

5). An abrupt increase in the synthesis of both cholesterol and fatty acid during the second hour of fasting as indicated by an 8.5 fold increase in the incorporation of label into fatty acid and a 2.8 fold increase into cholesterol as compared to the respective 12 hr fasted incorporation levels.

6). This greater increase in fatty acid labeling than cholesterol labeling results in a decrease in the 12 hr fasted Chol:FA specific activity ratio of 37:1 to a ratio of 13:1 at the end of the second hour of fasting.

7). A gradual decline in both cholesterol and fatty acid synthesis following the peak of synthetic activity observed at the 2 hr fast interval.

8). A fall in label incorporation into cholesterol to a level at the sixth hour of fasting 33% less than that observed in the 12 hr fasted animal.

Comparison of In Vitro and In Vivo Fasting Studies

As discussed at the first of this section, a study similar to the above reported in vitro study has been reported for the intact animal by Cockburn.[60] Since these intact animal studies report the % of an intraperitoneally injected "dose" of acetate label incorporated into total hepatic fatty acids and cholesterol while the present in vitro data are reported as the % of substrate acetate label incorporated into only one gram of liver slices, the two % incorporation figures are not strictly comparable. It is possible however to compare "normalized" % incorporation calculations as discussed above since these represent the response of fasted tissue to refeeding and subsequent fasting irrespective of the absolute incorporation figures.

Using this computation, it is possible to plot the in vitro and in vivo response curves, as has been done in Figures 9 and 10.

Changes in the synthesis of fatty acid and cholesterol in response to feeding and fasting are seen to differ both qualitatively and quantitatively when the in vivo and in vitro techniques are compared. These differences appear to be much greater for cholesterol than fatty acid.

Fatty Acids

The increased labeling of hepatic fatty acids subsequent to refeeding of the 12 hr fasted animal occurs much more rapidly in vivo than in vitro, reaching a value $4\frac{1}{2}$ times the fasted level during the 60 min feeding period as compared to rise of less than 50% in vitro. This initial

Figure 9

Time Course of Fasting

A comparison of the lipid labeling response in fatty acids to feeding and fasting as seen in the liver slice preparation and in the intact animal. Lipid labeling response = % incorporation at the indicated fast interval/% incorporation in the 12 hour fasted preparation. In vitro experiments are 1 gram samples of liver slices incubated for 1 hour at 37.5°C in 25 ml of Krebs-Ringer-Phosphate (pH 7.4) with 1 ml of acetate-1-C^{14} (0.253 uM and 100,000 cpm/ml).

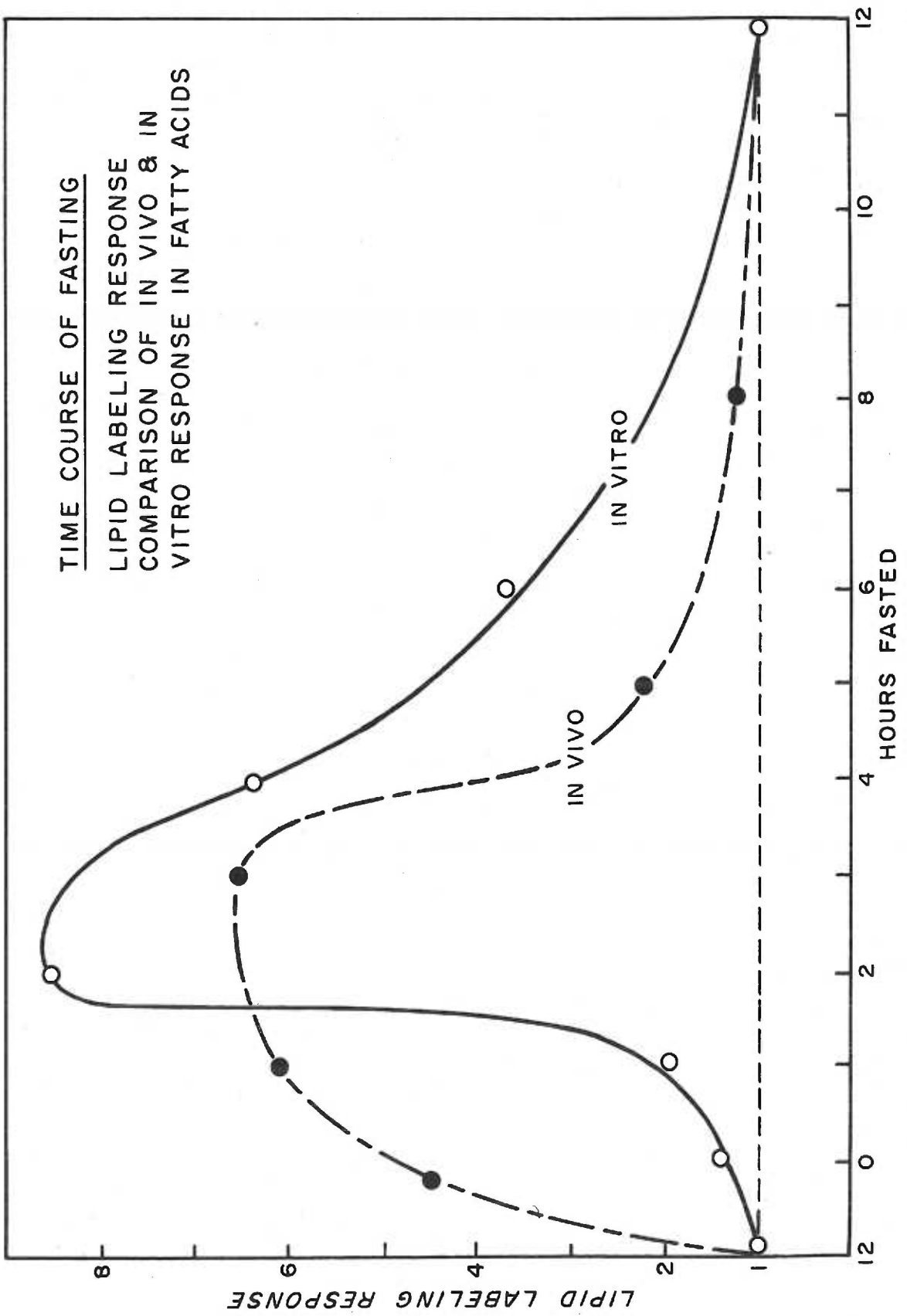
TIME COURSE OF FASTING
LIPID LABELING RESPONSE
COMPARISON OF IN VIVO & IN
VITRO RESPONSE IN FATTY ACIDS
IN VITRO
IN VIVO
LIPID LABELING RESPONSE
HOURS FASTED
12
10
8
6
4
2
0
12
2
4
6
8

FIGURE 9

Figure 10

Time Course of Fasting

A comparison of the lipid labeling response in cholesterol to feeding and fasting as seen in the liver slice preparation and in the intact animal. Lipid labeling response = % incorporation at the indicated fast interval/% incorporation in the 12 hour fasted preparation. In vitro experiments are 1 gram samples of liver slices incubated for 1 hour at 37.5°C in 25 ml of Krebs-Ringer-Phosphate (pH 7.4) with 1 ml of acetate-1-C^{14} (0.253 uM and 100,000 cpm/ml).

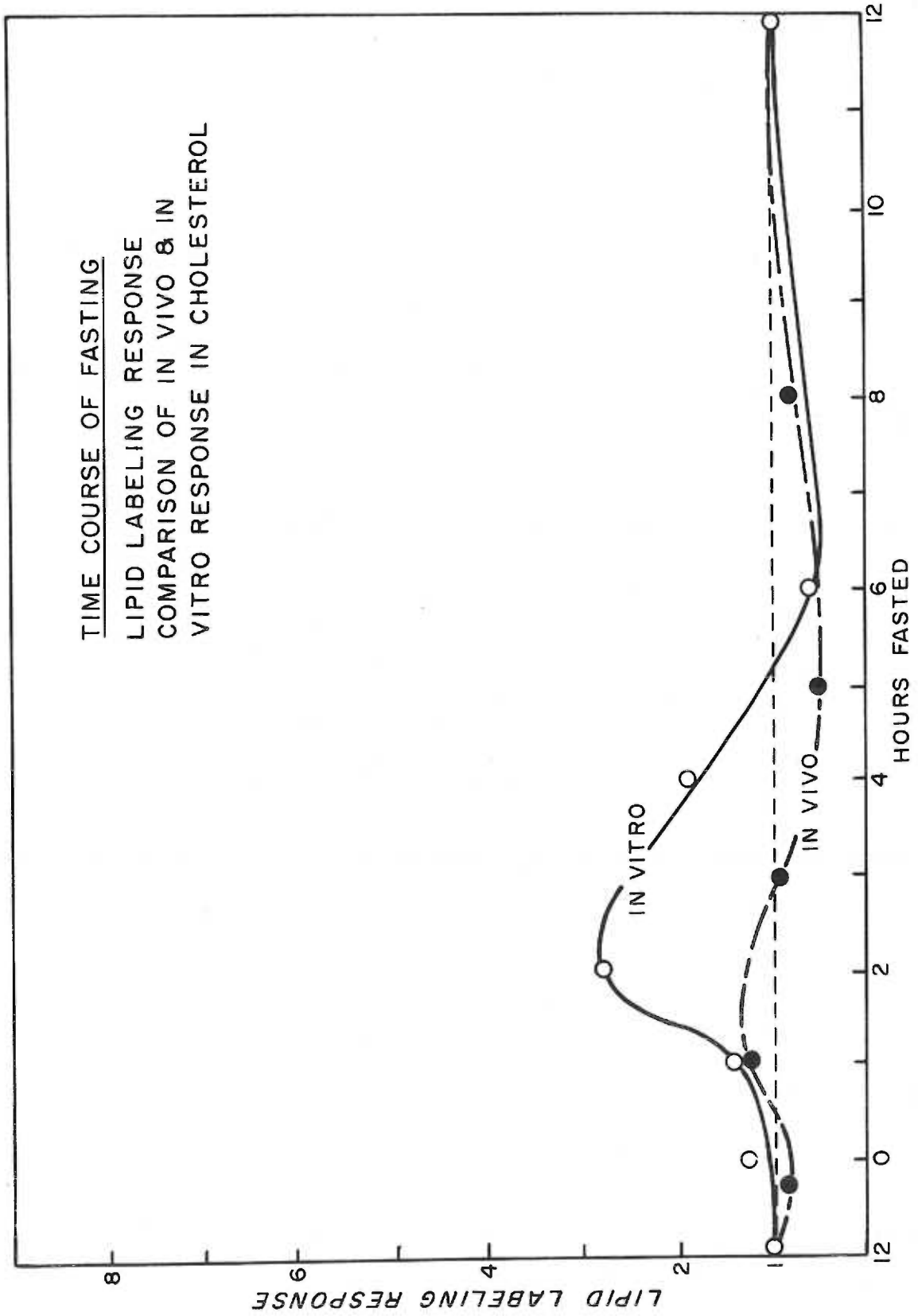
TIME COURSE OF FASTING
LIPID LABELING RESPONSE
COMPARISON OF IN VIVO & IN
VITRO RESPONSE IN CHOLESTEROL
LIPID LABELING RESPONSE
8
6
4
2
1
IN VITRO
IN VIVO
12
0
2
4
6
8
10
12
HOURS FASTED

FIGURE 10

rapidly increasing fatty acid synthesis in vivo continues to increase at decreasing rates up to the third hour of fasting, being nearly maximal one hour after feeding (6.1 fold increase at 1 hr as compared to 6.5 at 3 hrs). This is in contrast to the minimal increases observed in vitro during this initial one hour fast followed by an abrupt increase to the 2 hr fast maximum which is nearly $\frac{1}{3}$ greater than the maximum increase in vivo.

The decreasing synthesis of fatty acids seen in the later fasting periods also differs with the two techniques, being relatively abrupt in vivo and more gradual in vitro. By comparison, the 5 hr incorporation in vivo is only about twice the 12 hr incorporation while in vitro it is still nearly 4 times the 12 hr incorporation.

In vivo fatty acid labeling differs from in vitro labeling by: 1). an earlier initial increase with refeeding; 2). a sustained interval of increased synthesis; 3). maximum increase $\frac{1}{3}$ less than in vitro; 4). an abrupt fall in incorporation with 5). a return to nearly minimal labeling by the sixth or seventh hour of fasting.

Cholesterol

The differences in cholesterol synthesis observed between the two techniques are similar to those seen for fatty acid but more striking. In vivo, an initial drop in the % of the label incorporated into cholesterol has been consistently observed during the refeeding period. This decreased incorporation, averaging 15% less than the 12 hr fasted level, has been tentatively interpreted as an inhibition of hepatic "cholesterologenesis" by the influx of dietary cholesterol during the feeding period.

The initial depression of cholesterol synthesis is recovered rapidly and cholesterol labeling in vivo reaches a maximum one hour postprandial,

which represents only a 15% increase over the 12 hr fast level. There follows a gradually decreasing incorporation until the fifth postprandial hour when a minimum level is reached which is only slightly more than half the 12 hr level. The return to the 12 hr fast incorporation in vivo is gradual and constant during the remainder of the interval.

This in vivo cholesterol response is in contrast to the above reported in vitro response which by the second postprandial hour has increased nearly twelve times as much as the maximum increase observed in vivo. There is some similarity between the two systems in the gradual return to levels below the 12 hr fast incorporation, reaching a minimum 46% less than the 12 hr value in vivo and 33% less in vitro. This minimum incorporation is also observed at about the same time in the two systems (5 hr fast in vivo and 6-7 hr fast in vitro).

In vivo cholesterol labeling thus differs from in vitro by: 1). showing an initial depression in cholesterol synthesis during the feeding period; 2). increasing to a maximum incorporation only 15% greater than the 12 hr fast level (compared to a 176% increase in vitro); and 3). decreasing to a minimum incorporation 50% less than the 12 hr fast level by the fifth postprandial hr (compared to a 33% decrease at 6 hrs in vitro).

An additional difference between in vivo and in vitro data is marked by the use of these "normalized" incorporation figures. The actual % incorporation into cholesterol in the 12 hr fasted rat in vivo is 0.13% while for fatty acid it is 0.12%. By contrast, in vitro cholesterol incorporation is 1.35% compared to only 0.47% for fatty acid. Thus the % of the label actually incorporated into the two lipid fractions in the 12 hr fasted rat is nearly equal in vivo while in vitro there is nearly three times as much activity in cholesterol as is found in fatty acid.

Conclusions

To the best of our knowledge, this presentation is the first attempt to compare in vivo and in vitro systems in the field of lipid synthesis. From the above discussion it is evident that the two preparations are not identical. Useful comparative information on fatty acid formation might be obtained with either system but potentially invalid information on in vivo cholesterologenesis could be obtained with the in vitro system. It is entirely possible that in the absence of the homeostatic mechanisms thought to control cholesterol metabolism entirely misleading information may appear in the data.

The widespread use of the slice technique has yielded much important qualitative information. Attempts to calculate true rates of synthesis, etc., on the basis of slice data are becoming increasingly frequent. Our findings suggest that the slice preparation may yield false quantitative information.

Acetate Concentration Study

Introduction

Before arriving at final conclusions with regard to the differences between the in vitro and in vivo techniques as reported in the preceding sections, it was necessary to determine if the labeled acetate substrate in our in vitro experiments was of sufficient concentration to influence the rates of the reactions which it was tracing. In a survey of the literature only two reports were found which dealt with this subject; one by Medes, et al.[72] on the estimation of fatty acid synthesis rates and the second by Franz and Bucher[73] who reported on the effect of

increasing acetate concentration on the incorporation of acetate into cholesterol.

According to Medes, et al.,[72] an important proviso in using incorporation data to calculate total synthesis is that the substrate itself should have no influence on the reactions being measured. They state that "in all of the studies thus far carried out, labeled substrates have been used in concentrations higher than physiological" and considered the possibility that "the results of such experiments may not reflect the behavior of a substrate when present in low concentrations as a transient intermediate." It is also pointed out by these workers that "if a tracer is being used to determine a reaction rate, such as the rate of fatty acid synthesis, it is necessary to evaluate the effect of the tracer itself on the process. It is conceivable that the tracer substance may have varying effects on different reactions; consequently one may find different patterns of metabolic conversions at different substrate concentrations." In order to test how the metabolism of the acetyl group may be influenced by the concentration of precursors, these authors report a study of C^{14}-labeled lactate, glucose and acetate with regard to the effect of their concentration on their conversion to fatty acids, cholesterol, acetoacetate, and CO_2 in liver slices obtained from both fed and fasted rats. In each experiment aliquots of pooled liver slices were incubated with different concentrations of the labeled substances. Acetate (methyl-labeled) and DL-lactate (2-labeled) were studied at four concentration levels over the range, 0.00005 M to 0.05 M. The uniformly labeled glucose was studied at the three levels, 0.0005, 0.005, and 0.025 M.

The data that they present shows that for each of the four metabolic conversions being measured, there is a constant ratio of substrate concentration to incorporation of isotope resulting in a linear increase in the incorporation of labeled molecules into fatty acid, cholesterol, acetoacetate and CO_2 with increased substrate concentration. Moreover, the curves showing the incorporation into each of these four compartments are parallel, being linear at lower concentrations and tending to fall off slightly at higher concentrations. The authors interpret this fall off as being due to the inability of the enzymatic steps involved in formation of "active acetyl" to keep pace with the increase in concentration of the substrate. These investigators conclude that, "from the parallelism displayed by the curves for lipogenesis and ketogenesis, it is obvious that total fatty acid synthesis proceeds at a constant rate which is independent of wide ranges in substrate concentration."

Franz and Bucher[73] working with rat liver homogenates report findings which differ considerably from those of Medes, et al. In studies on the incorporation of acetate into cholesterol, they found that with increasing substrate concentrations, the rate at which the acetate was being oxidized to CO_2 remained fairly constant over the range of 10 to 50 uM of acetate and with increasing concentration there was a linear increase in the incorporation of label into CO_2. The incorporation of label into cholesterol was however linear only at the lower concentrations and at the 20 uM level a plateau was reached where no further increase in incorporation into cholesterol occurred, and in fact at the highest concentration level (50 uM) there was actually less

incorporation than at lower levels. These investigators thus showed a dissociation between the oxidation of acetate to CO_2 and its incorporation into cholesterol as the concentration of substrate acetate was increased; the incorporation into CO_2 continuing to rise while the incorporation into cholesterol reaches a plateau. No explanation of their findings is offered by these authors.

In view of the paucity of information on this subject as well as the divergent results which have been reported, we felt that further investigation of the problem was warranted and undertook the experiments reported below.

Experimental

A total of 18 rats were used in these studies. The first four were obtained from the Sprague-Dawley Farms, Madison, Wisconsin, the next seven from the colony of Sprague-Dawley rats maintained by the Department of Biochemistry at The University of Oregon Medical School, and the last seven were from the colony of Sprague-Dawley strain rats at The Northwest Rodent Company of Pullman, Washington. All were young adult male animals, carefully trained to feeding as before and used four hours following the standard one hour feeding period. Slice preparation, incubation, lipid separation, and radioassay were as previously described and incubation of the slices was for one hour.

Composition of Experimental Substrates—Since the range of acetate concentrations which could be studied with a single labeled acetate solution was limited by the sensitivity of our radioassay, it was necessary to use several dilutions of a stock acetate-1-C^{14} solution (solution number 104; 1 x 10^6 cpm and 2.53 uM acetate per ml = 0.5 mc per 25 ml) in

order to investigate the wide range of substrate concentrations reported. Two experimental methods were used as a control of the possibility that the various dilutions might not be identical. In the first study (A-1) the stock acetate solution (acetate #104) was diluted with water so as to contain 2.53, 0.253, 0.0253 and 0.00253 uM acetate per ml. Since there was no unlabeled acetate added the specific activity of each of the new solutions remained the same as that of the original solution (i.e., 400,000 cpm per uM) and thus the above solutions contained 1×10^6, 1×10^5, 1×10^4, and 1×10^3 cpm per ml respectively. This initial series of solutions was called A-1 and the individual solutions were labeled 1-a, 1-b, 1-c and 1-d respectively. These four solutions allowed a 1000-fold range of acetate concentrations. In order to extend this range unlabeled acetate, as sodium acetate, was added to an aliquot of the original stock solution of labeled acetate (#104) so as to increase the total acetate concentration of this solution ten-fold (i.e., from 2.53 uM to 25.3 uM per ml). This new solution, which had a specific activity of 40,000 cpm per uM acetate, was then diluted with water as above so as to obtain three additional solutions containing 2.53, 0.253 and 0.0253 uM acetate per ml. Since there was a ten-fold dilution of the acetate label and hence a specific activity one-tenth that of the solutions of the first series, the four solutions contained the same amount of activity per ml (1×10^6, 1×10^5, 1×10^4, and 1×10^3 cpm) despite the ten-fold increase in total acetate. This second series of solutions was called A-2 and the individual solutions were labeled 2-a, 2-b, 2-c and 2-d respectively. Two additional aliquots of the original acetate #104 solution were diluted with unlabeled acetate so as to provide solutions

containing 100-fold and 1000-fold increases in total acetate per ml and each of these new solutions was then diluted with water as above thus yielding two more series of dilutions, the first containing 4000 cpm per uM acetate and the second, 400 cpm per uM. The four solutions of the 4000 cpm series (series A-3) contained 253, 25.3, 2.53 and 0.253 uM acetate per ml and were labeled 3-a, 3-b, 3-c, and 3-d while those of the 400 cpm per uM series (A-4) contained 2530, 253, 25.3 and 2.53 uM acetate per ml and were labeled 4-a, 4-b, 4-c and 4-d respectively. As before, the number of counts remained constant, the first solution of each series containing 1×10^6 cpm per ml, the second, 1×10^5, the third, 1×10^4 and the fourth solution contained 1×10^3 cpm per ml. In this manner, four series of solutions were prepared, each containing the same amount of activity but with each representing a ten-fold increase in total acetate content and a ten-fold decrease in the specific activity of each succeeding series, (i.e., 400,000, 40,000,4000 and 400 cpm per uM acetate).

Aliquots of these dilutions, contained in 16 separate solutions, were used in the first eleven experiments which will be referred to as Study A. In each experiment four flasks were prepared, each of which contained one gm of liver slices, obtained from a single animal, suspended in 25 ml of Krebs-Ringer-Phosphate buffer. To the sidearm of each flask was added one ml of one of the acetate solutions so that each animal was used to study a single series of acetate dilutions, each with the same specific activity.

In the second group of experiments, study B, an entirely new series of solutions was prepared from the original stock acetate-1-C^{14} solution

(#104) in which the amount of activity was held constant in each series rather than the specific activity as in study A above. For the first series of solutions (B-1) an aliquot of solution #104 was diluted 1 to 100 to obtain a solution containing 0.0253 uM acetate and 1 x 10^4 cpm per ml. To aliquots of this solution (B-1-a) was added sufficient unlabeled acetate to make three new solutions containing 0.253, 2.53 and 25.3 uM acetate per ml respectively (solutions B-1-b, B-1-c and B-1-d), each containing 1 x 10^4 cpm per ml. Since each solution of this series contained successive ten-fold increments of unlabeled acetate the specific activities were 400,000, 40,000, 4000 and 400 cpm per uM acetate respectively. Similar treatment of a 1 to 10 water dilution of the original stock acetate #104 solution provided the second series of solutions (B-2) which contained 0.253, 2.53, 25.3 and 253 uM of acetate (solutions B-2-a, B-2-c and B-2-d respectively). Each of these solutions contained 1 x 10^5 cpm per ml and the same four specific activities as in series B-1. Finally, the addition of quantities of unlabeled acetate to undiluted aliquots of acetate #104 yielded the third series of solutions (B-3) containing 2.53, 25.3, 253 and 2530 uM acetate each with 1 x 10^6 cpm per ml and specific activities of 400,000, 40,000, 4000 and 400 cpm per uM respectively. These were labeled B-3-a, B-3-b, B-3-c and B-3-d.

A summary of the flask contents for each experiment is presented in Table XI. In all, a total of 72 one gm. samples of liver slices obtained from 18 rats was used to study a one million-fold range of acetate concentrations, extending from 0.00253 to 2530 uM of acetate

Table XI
ACETATE CONCENTRATION STUDY
Composition of Experimental Substrates

Study		uM C^{14}-Labeled Acetate*	uM Unlabeled Acetate	Total uM Acetate*	Cpm	SA**
A-1	a	2.53	0	2.53	$1x10^6$	400,000
	b	.253	0	.253	$1x10^5$	
	c	.0253	0	.0253	$1x10^4$	
	d	.00253	0	.00253	$1x10^3$	
A-2	a	2.53	22.80	25.3	$1x10^6$	40,000
	b	.253	2.28	2.53	$1x10^5$	
	c	.0253	.228	.253	$1x10^4$	
	d	.00253	.023	.0253	$1x10^3$	
A-3	a	2.53	250.5	253	$1x10^6$	4000
	b	.253	25.0	25.3	$1x10^5$	
	c	.0253	2.50	2.53	$1x10^4$	
	d	.00253	.250	.253	$1x10^3$	
A-4	a	2.53	2527.5	2530	$1x10^6$	400
	b	.253	252.8	253	$1x10^5$	
	c	.0253	25.3	25.3	$1x10^4$	
	d	.00253	2.53	2.53	$1x10^3$	
B-1	a	.0253	0	.0253	$1x10^4$	400,000
	b	"	.228	.253	"	40,000
	c	"	2.50	2.53	"	4000
	d	"	25.3	25.3	"	400
B-2	a	.253	0	.253	$1x10^5$	400,000
	b	"	2.28	2.53	"	40,000
	c	"	25.0	25.3	"	4000
	d	"	252.8	253	"	400
B-3	a	2.53	0	2.53	$1x10^6$	400,000
	b	"	22.8	25.3	"	40,000
	c	"	250.5	253	"	4000
	d	"	2527.5	2530	"	400

* All values are per 27 ml substrate volume.
** SA = specific activity in cpm per uM acetate.

added to the 25 ml substrate volume (9.4×10^{-8} to 9.4×10^{-2} molar with respect to acetate).

Results and Discussion

When a suitable tracer is added to a metabolic system that is carrying out a series of sequential synthetic steps it is to be expected that the tracer will be incorporated into a variety of compounds. If the tracer is present in small amounts, i.e., trace amounts, and if the metabolic sequence does not select between tracer and non-labeled precursor it is to be expected that as the amount of label is increased there will be a proportional increase in the amount of label incorporated into the intermediates or products. This is to say that changes in the specific activity of the precursor "pool" will be reflected proportionally in the magnitude of the labeling of the product. Should the amount of tracer be increased to the extent that the concentration of the precursor changes the metabolic equilibrium, variable increases in the labeling of the products may occur. A test for "true" tracer conditions then is to increase the amount of tracer and to follow the increase in specific activity of the product.

Data obtained on the gaseous exchanges of the systems are presented in Table XII. A small steady rise in O_2 utilization amounting to a total change of about 20% is seen for the entire range of acetate concentrations. The amount of CO_2 on the other hand did not show changes of this magnitude, causing a shift in the RQ values. The amount of acetate oxidized to CO_2 decreased at the 25 uM acetate level from its mean of 10.3% to 3.7 and 0.42% at the two higher acetate concentration levels.

Table XII
ACETATE CONCENTRATION STUDY
GAS EXCHANGE AND CO_2 % INCORPORATION
uM O_2 or CO_2/gm. tissue/hr

uM Acetate	O_2 uM/gm./hr	CO_2 uM/gm./hr	RQ.	CO_2 % Incorporation*
.00253	56.7	50.9	0.90	9.4
.0253	55.4	48.1	0.87	10.4
.253	53.8	48.2	0.90	10.2
2.53	57.5	48.9	0.85	10.9
25.3	59.9	52.5	0.88	10.6
253.0	69.0	56.4	0.82	3.7
2530.0	70.0	60.2	0.86	0.42

*S. A. CO_2 in c.p.m/uM x uM produced x 100 ÷ c.p.m. dose.

The CO_2 specific activity data obtained from the two studies is reported in Table XIII and that of the two lipid fractions in Tables XIV and XV.

Individual values have been reported to show the very narrow range of CO_2 specific activities compared to the wide range of lipid specific activities found in the same experiments. As has been pointed out in previous discussions, it is not uncommon to find five- and six-fold differences within a group of values, especially when lipid labeling is of a high order of magnitude. This variation is greatest in the fatty acid fraction, the lipid compartment known to be most sensitive to changes such as fasting[61,63] and experimental diabetes.[74]

Evaluation of these data can best be made by considering the above mentioned premise, i.e., increases in the specific activity of a precursor pool will be reflected in proportional increases in the specific activities of the various products of that pool, providing that the label introduced into the "pool" does not materially change the concentration of it. A graph of product specific activity plotted against substrate specific activity will quickly reveal any deviations from this constant ratio and thus, deviations from "true" tracer conditions. Figure 11 presents such a graphical evaluation of the data of Tables XIII, XIV, and XV. Rather than plotting actual specific activity figures for the various products, these data are plotted as the percent of the anticipated response, that is, the percent of the specific activity which would have been found had the change in the product specific activity been proportional to the magnitude of the substrate specific activity increase. Thus, a ten-fold increase in product specific activity with a ten-fold increase in substrate activity would represent 100% of the anticipated response. This situation is

TABLE XIII
ACETATE CONCENTRATION STUDY
Specific Activity of CO_2
(Cpm/uM CO_2)

Substrate SA*	400,000				40,000				4000				400			
Acetate Concentration**	A+		B+		A		B		A		B		A		B	
	SA‡	$\bar{X}$‡	SA	$\bar{X}$	SA	$\bar{X}$	SA	$\bar{X}$	SA	$\bar{X}$	SA	$\bar{X}$	SA	$\bar{X}$	SA	$\bar{X}$
.0025	199 182 154	178														
.025	183 188 168	179	238 206	222	391	391										
.25	211 184 155	183	225 263	244	151	151	228 214	221	153	153						
2.5	191 171 188	183	251 118	184	186	176	220 267	243	157 102 231	163	237 219	228	643 562	602		
25.0					150	150	218 58	138	140 190 267	199	193 186	189	614 508	561	231 250	247
250									39 57	48	64 36	50	70 68	69	54 68	61
2500													6.7 7.1	7.0	3.4 18	11

* Cpm/uM acetate
** uM acetate/27 ml substrate volume
\+ Experimental study A or B described in text
‡ Cpm/uM CO_2
‡ Mean specific activity

TABLE XIV
ACETATE CONCENTRATION STUDY
Specific Activity of Fatty Acid
(Cpm/mg. Fatty Acid)

Substrate SA*	400,000				40,000				4000				400			
Acetate Concentration**	A+		B+		A		B		A		B		A		B	
	SA‡	X̄‡	SA	X̄	SA	X̄	SA	X̄	SA	X̄	SA	X̄	SA	X̄	SA	X̄
.00253	228	175														
	65															
	293															
.0253	215	182	135	105	204	204										
	57		76													
	275															
.253	207	157	112	98	153	153	102	86	510	510						
	61		83				70									
	205															
2.53	116	114	88	64	95	95	94	77	402	185	87	77	45	35		
	35		40				60		71		67		24			
	191								82							
25.3					30	30	42	30	188	85	49	41	11	7	29	26
							19		35		32		2.4		22	
									32							
253									50	29	9.5	6.4	16	22	9.3	9.5
									8.6		3.3		28		9.7	
2530													2.4	3	1.5	1.3
													4.0		1.0	

* Cpm/uM acetate
** uM acetate/27 ml substrate volume
+ Experimental study A or B described in text
‡ Cpm/mg. fatty acid
‡ Mean specific activity

TABLE XV
ACETATE CONCENTRATION STUDY
Specific Activity of Cholesterol
(Cpm/mg. Cholesterol)

Substrate SA*	400,000				40,000				4000				400			
Acetate Concentration**	A+		B+		A		B		A		B		A		B	
	SA‡	X̄‡	SA	X̄	SA	X̄	SA	X̄	SA	X̄	SA	X̄	SA	X̄	SA	X̄
.00253	2266	2260														
	2348															
	2166															
.0253	1686	1370	1280	1890	1600	1600										
	1626		2500													
	798															
.253	1342	1179	1280	993	1175	1175	1000	1730	2090	2090						
	1636		706				2450									
	561															
2.53	821	680	686	753	727	727	1026	819	982	874	833	1380	824	1290		
	839		821				613		746		1920		1760			
	378								896							
25.3					218	218	261	300	337	364	433	364	123	130	333	526
							339		331		294		137		718	
									424							
253									50	50	36	44	39	57	64	65
									51		53		75		65	
2530													4.8	6	4.0	5.1
													8.5		6.1	

* Cpm/uM acetate
** uM acetate/27 ml substrate volume
\+ Experimental study A or B described in text
‡ Cpm/mg. cholesterol
‡ Mean specific activity

Figure 11

Acetate Concentration Study

Response to CO_2, fatty acids and cholesterol specific activities to increasing substrate acetate concentrations (both uM acetate and cpm). A ten-fold increase in product specific activity accompanying a ten-fold increase in substrate concentration (and activity) is termed a 100% response. Lesser responses in product specific activity are given as the percent of the response which would have been anticipated, had the product label increased proportionately to the increase in substrate concentration and activity. CO_2, O————O; fatty acids, x- - - - -x; cholesterol, Δ-·-·-·-·-Δ.

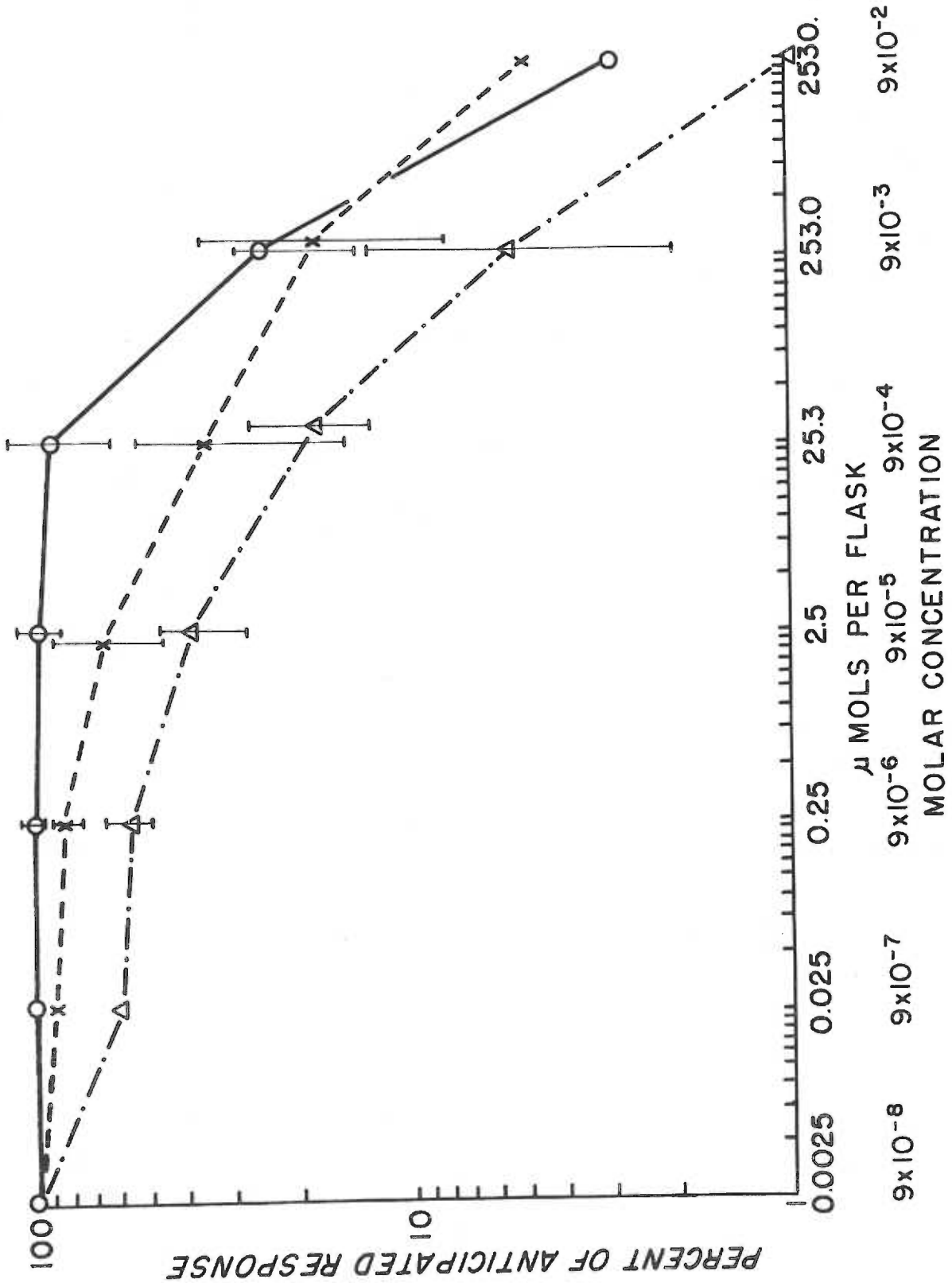
PERCENT OF ANTICIPATED RESPONSE
100
10
1
0.0025
0.025
0.25
2.5
25.3
253.0
2530.
μ MOLS PER FLASK
9x10-8
9x10-7
9x10-6
9x10-5
9x10-4
9x10-3
9x10-2
MOLAR CONCENTRATION

FIGURE II

well illustrated by the solid line representing the CO_2 response. The initial point on this line, i.e., 0.0025 uM acetate, is considered unity. At 0.025 uM (ten times the initial substrate concentration) the CO_2 specific activity was ten times that previously observed. In this way the CO_2 forming system is seen to respond in this linear fashion from 0.0025 to about 25 uM, a ten thousand-fold increase in substrate acetate. It is apparent then that up to 25 uM per flask (9.4×10^{-4} molar) the reactions involved in the oxidation of acetate to CO_2 are uninfluenced by the amount of "tracer" which must then have been accepted into the metabolic sequences without disturbing existing reaction rates, within the limits of the measurements used. Up to this concentration of acetate, it is difficult to determine the effect of substrate concentration on the volume of CO_2 formed because the small changes that might be anticipated (0.0005 to 5 uM of CO_2, assuming 10% of substrate acetate oxidized to CO_2) are well within the normal range of values usually encountered. The 25 uM level represents not only the last point of linear labeling response but also the last point at which the system could handle additional acetate as shown by the total CO_2 formation data.

As discussed by Medes, et al.,[72] this may represent the point at which the enzyme system or systems responsible for activation of the exogenous acetate to metabolically acceptable acetyl coenzyme A are no longer able to keep pace with the increasing demands being placed on them. Since the oxidation of acetate to CO_2 involves some 15 enzymatic reactions (i.e., one and one-half turns of the Krebs cycle), it is possible that the changes seen in the CO_2 data at the 25 uM level may be due to alteration of reactions other than acetate activation.

The specific activity response data for the lipid fractions reveals that these systems are grossly more sensitive to the amount of tracer present. At the 0.025 uM level, fatty acid activity is only 90% of that expected and at the 25 uM level the response is only 35% of the anticipated response. The lowest value, 5% at the 2500 uM level, is of the same order as that seen for CO_2 at this level. The cholesterol responses show by far the greatest deviations from the anticipated responses. From the lowest practical concentration level, 0.0025 uM, up to the first ten-fold increase, 0.025 uM, the response was only 60% of the expected response. At 250 uM the response was only 5% and at the 2500 uM level so little activity was present in the cholesterol fraction that precise radioassay was difficult. From these data it would appear that the concentration of tracer is of critical importance for the cholesterol forming system. This system appears to have little ability to increase its synthetic capacity when the substrate concentration of acetate is increased, even by amounts which would be universally acceptable as "tracer" amounts.

It is interesting to speculate on the reasons for the effects reported above and to attempt to pinpoint the metabolic step or steps limiting the specific activity responses. As discussed earlier, the first known step in tracer utilization involves entrance of the tracer acetate into the metabolic reactions by an activation process. Since this step is common to all reactions studied and since the CO_2 forming system responded to acetate increases in a linear fashion up to 250 uM, it is unlikely that the activation of acetate was the rate-limiting step for the lipid reactions. Should the acetyl-CoA formed not be common to each of the three metabolic pathways then activation of acetate for

fatty acid and/or cholesterol synthesis could be rate-limiting. Only one other point in the scheme of acetate metabolism (cf. Figure 1) appears to be common to the two lipid systems, i.e., the formation of acetoacetyl-CoA. It was unfortunately not possible in these studies to follow the formation of this C_4 lipid precursor. Of interest here is the ketogenic nature of the liver slice preparation as shown by Medes, et al.[72] It could be reasoned from this that the influx of exogenous acetate would tend to accumulate as acetoacetate which the liver appears incapable of re-activating for further metabolic utilization. In the absence of an adequate transport system to drain off this excess metabolite it may achieve levels toxic to other cellular metabolic processes.[10]

It appears that our data are similar to the findings of Franz and Bucher[73] mentioned earlier, although in homogenates of rat liver they found the incorporation of acetate into cholesterol to be fairly uniform up to a concentration of about 8×10^{-3} molar at which point a plateau was reached where no further activity appeared in the cholesterol fraction whereas our data shows the incorporation of activity into cholesterol to be non-linear with acetate concentrations as low as 9.4×10^{-7} molar. The obvious differences between the two experimental systems could easily account for this difference. Our data are however in sharp contrast with both the data and conclusions of Medes, et al.[72] Although we have studied substrate concentrations similar to those reported by these investigators (5×10^{-5} to 5×10^{-2} molar), using essentially the same experimental technique, we have been unable to confirm their findings of linear and parallel responses for CO_2, fatty acids and cholesterol throughout most of this range.

The significance of our data is apparent when one attempts a correlation of in vitro and in vivo studies where rates of synthesis are being compared. The lack of a uniform response for the two lipids in vitro may easily yield data very difficult to interpret and lead to some of the differences reported between in vivo and in vitro studies on such problems as experimental diabetes. At any rate, it indicates that a reappraisal of the whole concept of "tracer amounts" may be in order, especially with regard to the large volume of data being reported in which acetate is used as the label.

Summary

A total of 18 animals was used to study the influence of substrate acetate concentration on hepatic lipogenesis. By using acetate solutions of decreasing specific activity it was possible to investigate substrate concentrations ranging from 0.00253 to 2530 uM per 27 ml substrate volume, a one million-fold range. The results have been reported as percent of anticipated response which indicates the proportion of the increase in substrate acetate concentration (and activity) which is represented by the observed change in the incorporation of activity into the tissue fraction in question. Thus a ten-fold increase in product specific activity with a ten-fold increase in substrate acetate is called a 100% response. The following results have been reported:

1. The labeling response for CO_2 is linear from substrate concentrations of 0.00253 to 25.3 uM acetate per 27 ml substrate volume. Above this concentration, the response declines rapidly so that at the 253 uM level the response is only about 25% of that anticipated and at 2530 uM substrate acetate the response is less than 3%.

2. Fatty acid responses are nearly linear at the lower concentration levels (90% at 0.0253 uM and 80% at 0.253 uM) and fall off at higher levels, being 65% at 2.53 uM, 35% at 25.3 uM, 17% at 253 uM and 5% at the highest level of 2530 uM.

3. Cholesterol is seen to respond in a non-linear fashion at all levels of acetate studied. At the first ten-fold increase in substrate acetate concentration, the cholesterol response is only 60% of the anticipated response. At the 2.5 uM level the cholesterol response is only 35%, at 25.3 uM it is 18%, at 253 uM it is 5% and at the 2530 uM level virtually no activity was recovered in the cholesterol fraction.

4. A discussion of the possible localization of the metabolic step or steps in metabolism of acetate to CO_2, fatty acid and cholesterol which might be involved in this dissociation of labeling responses is presented.

5. It is concluded that a reappraisal of the concept of "tracer amounts" is indicated by our results.

CHAPTER IV

SUMMARY STATEMENTS

1. In an effort to correlate in vivo data on lipogenesis, obtained in this laboratory, with in vitro data as reported in the literature, a standardized liver slice technique has been developed which incorporates as many of our in vivo techniques as possible, including animal pretreatment, preparation and use of isotopic material, tissue fractionation, and radioassay.

2. Using this technique, certain in vivo experiments have been simulated in vitro and the data reported. In addition several other factors affecting lipogenesis, not previously investigated in this laboratory, have been studied in vitro. This includes the time course of incubation and the effect of tracer acetate concentration studies reported in this thesis.

3. Using slices obtained from the livers of fed and fasted rats, the effect of duration of incubation on lipogenesis was studied. During the course of incubation, there was a gradual decrease in the rate at which the label is incorporated into CO_2, fatty acids and cholesterol; this decrease being greatest for fatty acids (63% less at the end of 3 hours incubation), and less for CO_2 (27%) and cholesterol (33%). This decrease was, however, largely independent of the previous nutritional status of the animal, the slopes being similar in both the fed and fasted animals. That there was an actual difference in the nutri-

tional status of the two series of animals was shown by the actual percent incorporation data for any given period of incubation. Thus, "fed" slices incorporated 23% more label into fatty acid than did "fasted" slices and 78% more label into cholesterol.

4. A total of 36 rats were used to study the effect of fasting and refeeding on hepatic lipogenesis. No changes were found in O_2 utilization, CO_2 production or labeling of CO_2 following refeeding and subsequent fasting of 12 hour fasted animals. In the fasted rat, there was nearly three times as much incorporation of label into cholesterol as into fatty acids and nearly 37 times as much incorporation per mg. of cholesterol as per mg. of fatty acid. A gradually increasing incorporation of label into fatty acid and cholesterol was noted during the 60 minute feeding period and the first hour of fasting, representing a 25% increase for cholesterol and a 96% increase for fatty acid. An abrupt increase was seen in the synthesis of both cholesterol and fatty acid during the second hour of fasting as indicated by the 8.5-fold increase in the incorporation of label into fatty acid and the 2.8-fold increase into cholesterol. A gradual decline in both cholesterol and fatty acid synthesis followed the peak synthetic activity observed in the two hour fasted preparation. This decline in activity was proportionately greater in cholesterol, resulting in an incorporation in the six hour fasted slice which was 33% less than that seen in the twelve hour fasted preparation.

5. A comparison of the influence of refeeding on hepatic lipogenesis in vivo and in vitro was made. It was found that in vivo fatty acid labeling differed from in vitro labeling by showing: an earlier

initial increase with refeeding; a sustained interval of increased synthesis; a maximum increase $\frac{1}{3}$ less than in vitro; an abrupt fall in incorporation with a return to nearly minimal labeling by the sixth or seventh hour of fasting. In vivo cholesterol labeling differed from in vitro by showing: an initial depression in cholesterol synthesis during the feeding period; an increase to a maximum incorporation only 15% greater than the 12 hour fast level compared to a 176% increase in vitro; a decrease to a minimum incorporation 50% less than the 12 hour fast level by the fifth postprandial hour compared to a 33% decrease at six hours in vitro.

6. A study was made of the influence of the substrate concentration of acetate on lipid labeling. Contrary to the prevalent opinion that the concentration of this labeled substrate may be varied over a relatively wide range without influencing its incorporation into lipids, it was found that each of the fractions studied (CO_2, fatty acids and cholesterol) behaved in a different fashion to increasing amounts of substrate acetate. Each failed to show proportional increases in incorporated activity with substrate activity increases at definite substrate acetate concentrations. The incorporation of acetate activity into CO_2 increased linearly with linear increases in substrate acetate concentration (and activity) from acetate concentrations of 0.00253 uM per 27 ml substrate volume to 25.3 uM. A similar linear response was seen for fatty acid systems extending from the lower substrate concentration level of 0.00253 uM up to the 0.0253 uM level. Further increases in substrate concentration resulted in more and more marked deviations from a linear response so that at the 25.3 uM level only 35% of the

anticipated response was seen. Cholesterol systems failed to respond linearly at any concentration level, being only 60% of the anticipated response at the 0.0253 uM level (compared to 100% for CO_2 and over 90% for fatty acid), and only about 20% at the 25.3 uM level. It was concluded that a reappraisal of the concept of "tracer amounts" is needed in view of our data.

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