

1942

## William C. Stadie, 1942

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# INTERMEDIARY METABOLISM IN DIABETES MELLITUS<sup>1</sup>

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IN THE study of diabetes mellitus experiments have been initiated by and centered around two great discoveries. The first was that of v. Mehring and Minkowski, who, by removal of the pancreas, first produced in experimental animals a condition closely resembling diabetes mellitus in man. The second was that of Banting and Best whose discovery of insulin made it possible to restore the diabetic animal to normal by the injection of a few milligrams of a highly purified protein. Around the first discovery there accumulated a great literature out of which there emerged a chemical picture of the disturbed metabolism of the diabetic. Out of the second there grew a problem, namely the problem of the chemical action of insulin. It proposes the questions: "By what chemical mechanisms does insulin produce its striking metabolic effects?" or "In what specific chemical reactions of the intermediary metabolism does insulin engage?" In contrast to the first field, the literature attempting to answer these questions is surprisingly small.

About three years ago I became interested in this latter problem. The experimental work (1, 2, 3, 4, 5, 6, 7) which was then begun was done in collaboration with Dr. John A. Zapp, Jr., of the Department of Research Medicine, and Dr. Francis D. W. Lukens of the Cox Institute.<sup>2</sup> To them I wish to acknowledge my warm appreciation for their unstinting cooperation. In this paper I shall discuss some of these experiments. (It will be

<sup>1</sup> Lecture delivered January 15, 1942.

<sup>2</sup> For financial aid in support of the experimental work reported in these papers acknowledgment is made to the following Foundations: The Rockefeller Foundation, the American Philosophical Society, The Committee on Research in Endocrinology of the National Research Council, the Ella Sachs Plotz Foundation, and the Faculty Research Committee of the University of Pennsylvania.

understood, of course, that limitation of space makes it impossible for me to mention many important and significant papers by other workers in this field.)

It seemed to me, however, that a direct attack upon the problem of chemical action of insulin was premature, for the interpretation of the general chemical picture of diabetes mellitus was still a matter of sharp dispute. Students of the disease were divided in advocating two contending theories of diabetes. On the one side we find the under-utilizationists. They constitute the large majority and their thesis is the more popular one. It holds that the main, if not the sole, defect in the intermediary metabolism in diabetes mellitus is one in which the peripheral tissue (i.e., chiefly muscle) cannot either at all or in sufficient measure, oxidize carbohydrate without the catalytic intervention of insulin. On the other side is a smaller group—the over-productionists. Their thesis is that there is an overproduction of carbohydrate by the liver chiefly from fatty acids which are convertible into carbohydrate. The function of insulin is to control, directly or indirectly, the extent of this conversion; its action in the periphery to catalyze the oxidation of carbohydrate, is either nil or of minor importance.

In the light of either theory the complete diabetic is in a bad way. According to the under-utilizationists no energy is derivable from carbohydrates, about 60–80 per cent of that from protein is lost, hence fat must form the bulk of his metabolic mixture. According to the over-productionists the diabetic is wasting potential foodstuffs by excreting large amounts of fat in the form of sugar into the urine. In either case the question of fat metabolism assumes major proportions and until we are well oriented on this question the problem of the chemical action of insulin must remain obscure. It is necessary then to examine the hypothesis of fat metabolism which grew up simultaneously with the development of the above hypotheses of the diabetic defect.

In the light of our present knowledge of fat metabolism it is possible, I believe, to include all conceivable hypotheses of fat metabolism in the two following chemical mechanisms (Fig. 1):

Conceivable chemical mechanisms of fat metabolism.

I—Direct utilization by peripheral tissues.

Oxidation is both initiated and completed in the periphery.

II—Indirect utilization.

Preliminary partial oxidation in liver to diffusible, oxidizable substances which are utilized by the periphery.

FIG. 1.

I shall discuss later reasons for believing that both of these mechanisms are operative at the same time.

Concerning the first mechanism, that is, the direct utilization of fat in the periphery, almost nothing is known. The literature contains few experiments which shed light upon the mechanisms involved. In sharp contrast, we find the intermediary metabolism of carbohydrates by the muscle to be fairly well mapped out; yet a similar map of the intermediary metabolism of fat in the muscles is virtually blank.

Concerning the second mechanism, i.e., the preliminary partial oxidation of fats in the liver, we are more fortunate, and indeed, it is this mechanism which I shall chiefly discuss tonight. It is here, however, that differences of opinion arose and split the diabetic camp into two. The details of the contending hypotheses which have been advocated are summed up in Fig. 2.

Contending hypotheses concerning the preliminary oxidation of fatty acids in the liver.

II—A. Fatty acids oxidized to ketone bodies + acetic acid.

II—B. Fatty acids oxidized to ketone bodies + glucose.

II—C. Fatty acids oxidized to ketone bodies only.

FIG. 2.

According to the first of these, II—A, fats undergo a preliminary partial oxidation in the liver to a four carbon ketone residue, the balance of the molecule being oxidized to a two-carbon compound, i.e., acetic acid. In the normal both of these substances are freely oxidized in the periphery, but in the diabetic, the oxidation of the ketone residue cannot occur. This represented the position of the under-utilizationists.

According to the second hypothesis, II—B, fatty acid likewise

undergoes partial oxidation in the liver but the products of this oxidation are glucose and ketone bodies. This was the contention of the over-productionists.

The third hypothesis, II-C, postulates that the partial oxidation of the fatty acids in the liver results in the formation of ketone bodies only. This position represents, indeed, a new departure and is the one for which experimental evidence will be given.

It is interesting to note the influences which focused attention upon the first of these hypotheses and obscured the others. Among these were: first, the ascendancy of the under-utilizationists led by Lusk; second, the entrenched position of the Knoop hypothesis of successive beta oxidation of fatty acids so strongly advocated by Embden and by Dakin; and lastly, the development by Woodyatt and by Shaffer of the hypothesis of obligatory coupling of ketone-body-carbohydrate oxidation in the periphery. It became necessary to examine these last two hypotheses before proceeding further.

Current hypotheses of fat metabolism in the diabetic.

I—Knoop hypothesis of successive beta oxidation.

Long carbon chains of fatty acids oxidized two carbons at a time with formation of acetic acid + one ketone molecule per molecule of fatty acid.

E.g. Palmitic +  $6.5 \text{ O}_2 = 1 \text{ ketone} + 6 \text{ acetic}$ .

II—Ketones oxidized only by obligatory coupling with carbohydrate oxidation.

III—Fat converted to carbohydrate by liver.

FIG. 3.

Knoop's original experiments with phenyl substituted fatty acids showed from the nature of the phenyl residue excreted in the urine, that the short fatty acids which he used were oxidized at the carbon atom which is in the beta position to the terminal carboxyl group. In the case of the five carbon fatty acid, the longest chain used, Knoop found delta as well as beta oxidation. He made no statement about the possible splitting off of a two carbon compound such as acetic acid, nor did he conclude that

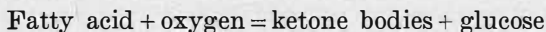
this beta oxidation found with short fatty acids was representative of a general biological reaction applicable to all fatty acids. These experiments of Knoop were rapidly confirmed and extended notably by Dakin (8) and the experience with short fatty acids was generalized to include a biochemical reaction for the oxidation of all fatty acids. There were two characteristics of this oxidative reaction, namely, (1) oxidation at the carbon atom next but one to the terminal carboxyl group, i.e., beta oxidation, and (2) the splitting off of two carbon atoms presumably as acetic acid. The process became known by its full name, successive beta oxidation and as such became firmly fixed in the literature. According to it the oxidation of the long naturally occurring even numbered fatty acid chains containing 16 or more carbon atoms would proceed as follows: Successive molecules of acetic acid would be split off by beta oxidation leaving a series of fatty acid residues each shorter by two carbon atoms than its immediate precursor. Finally one molecule of butyric acid would result which in turn was oxidized to aceto-acetic or beta hydroxy-butyric acid. For every molecule of fatty acid oxidized only one molecule of ketone-body would be formed. This process was confined to the liver; there is no evidence that a similar process occurs in the periphery.

Along with the hypothesis of successive beta oxidation, another hypothesis concerning fat metabolism was developing. I have called this the hypothesis of obligatory coupling of ketone-carbohydrate oxidation. According to it there is a definite stoichiometric reaction between ketone and carbohydrate of such a nature that exactly one, or perhaps two, molecules of ketone reacts with one molecule of carbohydrate in such a way that when the carbohydrate is oxidized the ketone is simultaneously oxidized. If this coupled reaction cannot take place, either by lack of available carbohydrates, as in fasting, or lack of ability to oxidize carbohydrate, as in diabetes, then there is no way in which the ketone bodies can be oxidized and they accumulate or are excreted as toxic or waste products of fat metabolism.

The combination of these two hypotheses represented in the

main the position of the under-utilizationists. The complete diabetic losing most of the energy from protein and carbohydrate still has 50 per cent of the original energy of the fat in the form of acetic acid. Lacking ability to bring into play the obligatory coupled reaction with carbohydrate the diabetic excreted the residual ketone. To be sure, this was a bit wasteful of fat but unless the ketones were toxic, which most people agreed was not the case, the process would have been harmless were it not for the unfortunate fact that ketone excretion robbed the body of base thus threatening fatal coma. Further, the hypotheses avoided postulating the formation of carbohydrate from fat. Finally, the discovery of the ketogenic-antiketogenic ratio appeared to offer a quantitative explanation of the marked influence of carbohydrate utilization upon ketone excretion. There even accrued about this position a supporting aphorism, "The fats burn in the flame of the carbohydrates."

Meanwhile the position of the over-productionists was developing, but upon somewhat less well entrenched lines. Obviously they could hardly espouse the obligatory coupling hypothesis for then ketonuria would never occur since by assumption abundance of carbohydrate is oxidized in the periphery. They therefore occupied themselves with undermining this position of the under-utilizationists, and showed, by the work of Chaikoff and Soskin (9), for example, that the ketone bodies were oxidized in the periphery without coupling with carbohydrate oxidation. The abnormal fat metabolism in diabetes could be more easily explained by postulating that there occurred in the liver the following reaction:



The exact stoichiometrical relations of this reaction were never clearly defined. The important assumption was made that this reaction was controlled by insulin. In diabetes the control was lost and the reaction ran to excess with overproduction of both ketone bodies and glucose.

The possibility of the conversion of fat to carbohydrate continued to be fought for bitterly by its advocates but their argu-

ments brought little conviction to the under-utilizationists. Indeed, Lusk characterized the reaction as a "figment of the imagination" which "should be relegated to the realm of scientific superstition." However, the critical experiment which would decide the matter one way or another still remained to be done.

It seemed to me that the problem of the chemical action of insulin must be preceded by a flank attack upon the problem of fat metabolism in diabetes. Indeed, in retrospect of the literature, it was evident that the position held for a period of 30 to 40 years was already considerably undermined. For example, in the normal fasting animal or in the diabetic animal, who must be subsisting chiefly upon fats, there should be found in the liver large amounts of intermediate fatty acids with 14, 12, 10 or less carbon atoms. But they had never been found.

Again, consider the case of the working diabetic, particularly the exercising depancreatized dog. He should oxidize no carbohydrate, hence he could oxidize no ketones. Now exercise him and measure the extra calories produced. These extra calories must come from fat, and by the Knoop hypothesis, one could calculate exactly the extra ketones formed. These should all appear in the urine, but no extra ketones whatever were found as can be easily calculated from Barker's (10) recent experiments. The implication seems obvious: The extra fat was completely oxidized without benefit of coupled carbohydrate oxidation. This fact, namely that exercise did not increase the ketonuria of the complete diabetic, was completely at variance with the obligatory coupling hypothesis and should long ago have forced its abandonment.

The generalization of Knoop's experiments on short fatty acid chains to include an explanation of fat metabolism in the diabetic came under fire shortly after its publication. In 1916 Hurtley (11), an English clinician, published a comprehensive paper on ketosis in diabetics. He was struck by the fact that none of the lower fatty acids had been found in the livers of patients dying in coma. Particularly was this so in the case of butyric acid which should have been revealed by its odor alone. Hurtley had



no experiments but on theoretical grounds he proposed an alternative hypothesis, which became known as the multiple alternate oxidation hypothesis. On the basis of in vitro studies with liver slices Jowett and Quastel (12) brought it out again. The two hypotheses are contrasted in a schematic way in the figure (Fig.

### SCHEMATIC OXIDATION OF PALMITIC ACID BY DIABETIC LIVER

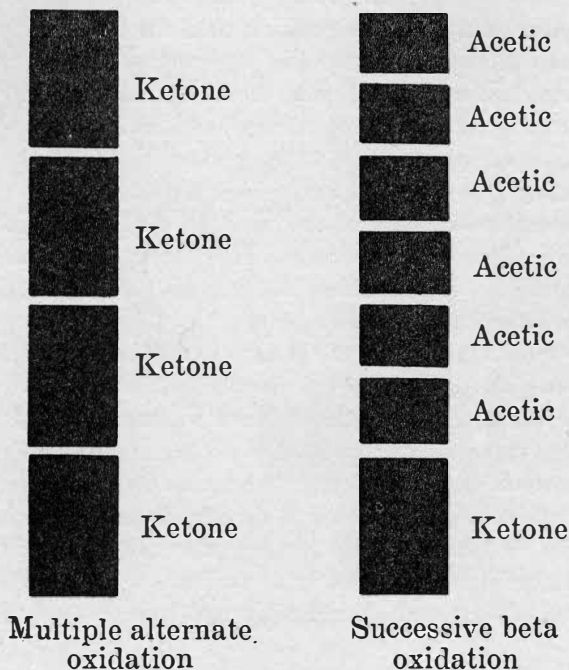


FIG. 4.

4). The multiple alternate oxidation hypothesis states that the long fatty acid molecule is oxidized along the entire length of the chain at alternate carbon atoms. The result is the complete disappearance of the fatty acid with the simultaneous appearance of an equivalent amount of ketone bodies. The Knoop hypothesis, on the other hand, supposes that there is a step by step oxidation

of the molecule. Paired carbon atoms are split off to form acetic acid leaving a residue of one molecule of ketone body. The contrasting chemical consequences of these two theories for the oxidation of, for example, palmitic acid, are as follows: (1) The production of one molecule of ketone should require  $1\frac{1}{2}$  molecules of oxygen or  $6\frac{1}{2}$ ; (2) for every molecule of fatty acid oxidized there should appear 4 molecules or only 1; (3) the oxidation of one molecule of fatty acid should give no acetic acid or 6 molecules. In our own experiments we attacked the problem in three

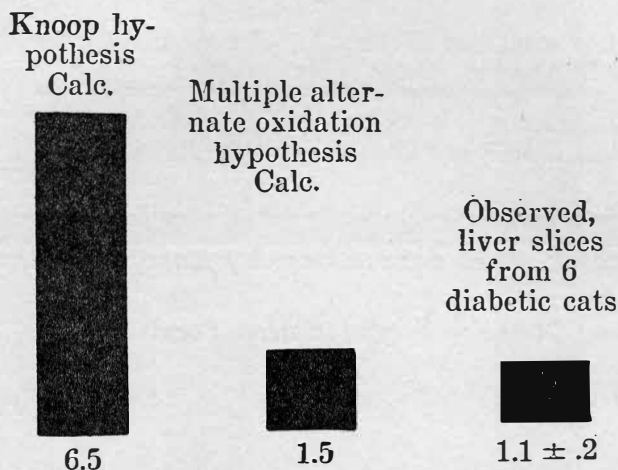




FIG. 5. The oxygen requirements (moles) for the production of a mole of ketone from palmitic acid by hepatic oxidation.

different ways each of which was designed to give a critical answer to the problem. In the first of these illustrated in the figure (Fig. 5), the oxygen requirements for the production of one mole of ketone body are given. For the multiple alternate oxidation hypothesis there would be required 1.5 while for the Knoop hypothesis the value would be 6.5. The answer to the problem should be found in the fat metabolism of the liver from the diabetic cat. This was measured in the following way: 2 to 4 days after pancreatectomy, the animal was sacrificed, and liver slices were prepared and equilibrated in vitro in suitable buffers

with 100 per cent of oxygen. Ketone formation and oxygen consumption were measured over a period of two hours. The results appear in the last column. We found (Fig. 5) in a series of 6 diabetic cats a ratio of oxygen consumption to ketone formation of  $1.1 \pm 0.2$  a value not significantly different from that required by the multiple alternate oxidation hypothesis. The ratio clearly excludes the Knoop hypothesis. In the second type of experiment (Fig. 6) we looked for the possible formation of acetic acid. These liver slices from diabetic cats were producing large amounts

Non-formation of Acetic Acid by Liver Slices from Diabetic Cat (No. 106B).

 *Observed hepatic ketones*

  
*Acetic acid formation by Knoop hypothesis*

| 0.0 *Observed acetic acid formation*

| 0.0 *Observed lower fatty acid (C<sub>2</sub>-C<sub>8</sub>)*

FIG. 6.

of ketone bodies in vitro as shown by the top block. The calculated acetic acid according to the Knoop hypothesis is indicated in the solid block. Yet in a series of animals we were unable to demonstrate any acetic acid either initially or at the end of two hours of equilibration in vitro. The possibility that the acetic acid was formed and then oxidized, was excluded by the determination of the respiratory quotient of the slices which in that case should have been about 0.7 instead of the value of 0.3 which was actually found. We also searched for the possible formation of the intermediary fatty acids containing one to eight carbon atoms which were supposed to form by successive beta oxidation but were unable to demonstrate even traces of them. The third

type of experiment (Fig. 7) is in essence a balance study of the fatty acid metabolism of liver slices from diabetic, normal phlorhizinized cats, and phlorhizinized rats. We would expect, according to the multiple alternate oxidation hypothesis that for every mole of fatty acid which is completely oxidized and therefore disappears from the liver, there would be formed four moles of ketones. That is to say, the fatty acid oxidized away should be replaced, as the slide shows, in the first and third blocks, by

### The Balance between Fatty Acids Oxidized and Ketones Formed by the Liver.

(Diabetic cats; phlorhizinized cats or rats.)

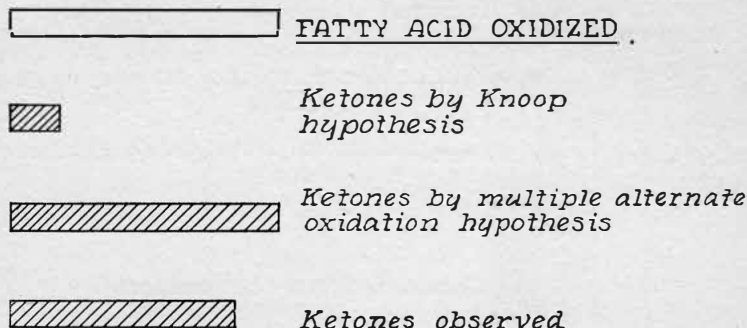


FIG. 7.

an equivalent amount of ketones. On the other hand, the Knoop hypothesis would predict the formation of only one fourth equivalents of ketones. In a series of eight animals we found as the last segment of the slide shows, that the decrease by oxidation of fatty acid during two hour equilibration of the liver slices in vitro was accounted for by the appearance of approximately an equivalent amount of ketone, a result in accordance with the multiple alternate oxidation hypothesis.

These experiments, along with other evidence in the literature,

led us to conclude that the successive beta oxidation hypothesis as applied to the oxidation of fatty acids in the liver could not be accepted and should be replaced by the multiple alternate oxidation hypothesis.

This conclusion must not be interpreted as meaning that beta oxidation does not occur at all in the liver. Indeed, Stetten and Schoenheimer (13) using deuterium have definitely shown that it does occur. It does mean, however, that the great bulk of fatty acid oxidation in the liver goes by way of multiple alternate oxidation rather than beta oxidation.

### The Oxidative Metabolism of Liver Slices from Six Diabetic Cats.

Mean oxygen/gm.liver/hr.  
Micromoles

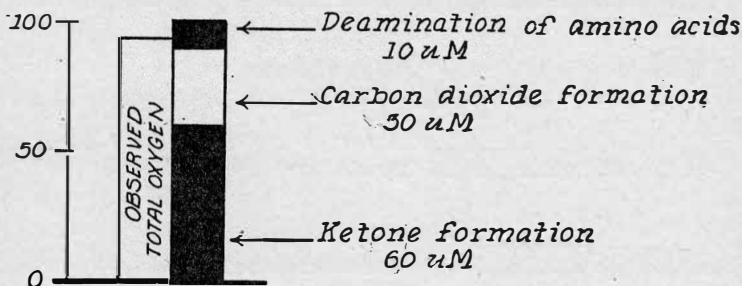


FIG. 8.

It is now possible to write the stoichiometrical equation for the oxidation of fatty acids in the liver of the diabetic and it is to be noted that no glucose is included in the reaction. However, the problem is of sufficient importance to warrant further experiment and I show two other types of evidence on this point. Glucose is comparatively rich in oxygen, namely 1 atom of oxygen per atom of carbon. Fatty acids on the contrary contain very little, about  $\frac{1}{8}$  of an atom per atom of carbon. If the diabetic liver converts fatty acids into glucose it must furnish by respiration

at least  $\frac{7}{8}$  of an atom of oxygen for every atom of fat carbon which it builds into the glucose molecule. Hence a balance sheet of the oxidative metabolism of liver slices from diabetic cats should yield significant information about the possibility of the conversion of fats to carbohydrates. Such a balance sheet was determined in the case of 6 depancreatized cats and is shown in the slide. Three known oxidative processes were independently measured and the oxygen required for each calculated (Fig. 8). Deamination of amino acids measured by urea formation required  $10 \mu\text{M}$  of  $\text{O}_2$  per gm. of liver per hour. Carbon dioxide formation required 30, and ketone formation required 60. The sum of these is  $100 \mu\text{M/gm. of liver/hr.}$  The total actually observed oxygen uptake was  $88 \mu\text{M/gm. of liver/hr.}$  not significantly different from the sum for the three known oxidative processes. In other words, within the limits of error, the balance was exact. But these same cats excreted 1 to 4 hours before the experiment with the liver slices, amounts of glucose which if it came from fat would have required at least  $97 \mu\text{M/gm./hr.}$  for this one oxidative process alone. But the experiment shows that there was no oxygen whatever available in the metabolism of the liver slices for this supposed synthesis. This experiment, alone, even if there were no others available, is strong proof that the conversion of fatty acids to glucose does not occur in the diabetic liver.

In the second type of experiment bearing on this point (Fig. 9), we measured the actual new carbohydrate formation by diabetic liver slices equilibrated in vitro. In addition we measured the protein metabolism by measuring urea formation and the fat metabolism (as ketone formation) in order to know how much glycerol might be available for carbohydrate formation. And lastly, we measured lactic acid metabolism in order to know its role in potential carbohydrate formation. In this way we could construct a balance sheet of carbohydrate precursors. These liver slices were actively oxidizing fats as shown by the abundant ketone formation and according to the hypothesis should also have been forming new carbohydrate from fat. But when the balance sheet was added up we could find no significant amount

FIG. 9.

*Synthesis of Fermentable Carbohydrate by Liver Slices from Diabetic Cats*

Equilibration for 2.0 hours at 38°; average sample 200 to 250 mg.; buffer 3.0 cc. of phosphate-saline or bicarbonate-saline at pH 7.2. Supplements as indicated.

The results are expressed in micromoles or microequivalents per gm. per 2 hours.

Cat No.	Supplement	Oxygen	R.Q.	Increase of ketone bodies	Increase of urea + NH <sub>3</sub>	Change in lactic acid	Increase of total carbohydrate	Increase of total carbohydrate corrected	Type of correction*
		<i>micro-moles</i>		<i>micro-moles</i>	<i>micro-equivalents</i>	<i>micro-moles</i>	<i>micro-moles</i>	<i>micro-moles</i>	
107CT	None	177	0.34	64	26	12.0	7.2	3.6	<i>N, K, L</i>
110AU	"	341*	0.32	170			15.5	1.5	" "
111EU	"	167	0.35		12	15.3	10.3	11.0	" L
111FU	"	185	0.33			17.9	9.9	11.9	" "
120BU	"	182	0.37	20	41	16.2	6.9	6.4	" K, L
120BT	"	159	0.38	20	41	3.0	1.7	-5.4	" " "
111AU	0.0005 M <i>D</i> -lactate	165	0.45	23		- 4.0	30.0	20.1	" " "
111BU	0.0025 " "	164	0.39	15		- 18.0	15.0	- 1.5	" " "
111CU	0.007 " "	221			30	- 40.0	27.0	0.1	" L
111DU	0.007 " "	209	0.53		22	- 78.0	54.0	8.1	" "
Mean					28				

## Equilibrated 4 hours. Summary\*

Samples	Type of correction	Mean corrected carbohydrate formation
5	<i>N, K, L</i>	4.6 ± 4.3
4	" <i>L</i>	7.8 ± 2.8
1	" <i>K</i>	1.5
10	Mean of all	5.6 ± 2.5 = 1.0 ± 0.5 mg. per gm. per 2 hrs.

\* Method of correction given in the text: *N* = correction for glycogenic amino acids; *K* = correction for glycerol from fat catabolized; *L* = correction for increase or decrease of lactic acid.

of new carbohydrate formation by the liver slices from the diabetic cats which could not be accounted for as coming from either catabolic protein, catabolic glycerol or lactic acid initially present in the liver.

In contrast to our inability to demonstrate formation of glucose from fat was our experience with a known precursor of car-

### METHOD I. KETONE UTILIZATION BY 8 DEPANCREATIZED CATS.

#### KETONES FORMED BY LIVER SLICES

Per Kg body weight per hour

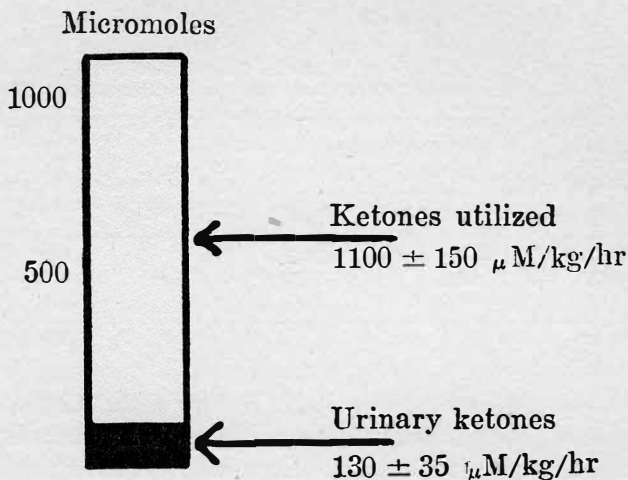


FIG. 10.

bohydrate. For example, these same liver preparations in the presence of lactic acid formed new carbohydrate up to 10 mg./gm. of liver/2 hrs. an amount sufficient to account for a large fraction of the glucose actually excreted by the cat in a preliminary period of observation.

These experiments convinced us that the diabetic liver was producing nothing but ketones by partial oxidation of fatty acids.



Neither acetic acid nor glucose was formed. It seemed clear that these ketone bodies must be oxidized in the periphery for otherwise there would be practically no source of energy available since most of the energy from protein and all of that from carbohydrate was out of the picture. The demonstration that the diabetic could utilize ketones peripherally became important and in five ways we showed that this was the case. Four of these were on animals and one on human cases of diabetes mellitus. I shall mention only two types of animal experiments.

In a preliminary period of 2 to 3 hours the urinary ketone excretion of diabetic cats was measured (Fig. 10). The animal was then sacrificed, the liver quickly removed and slices were pre-

FIG. 10A.

*Ketone Formation by Liver of Diabetic Cats, Calculated from Portal and Hepatic Blood Ketone Concentration in Intact Animal*

Cat No.	Blood ketones, micromoles per cc.		Calculated liver ketone formation, micromoles per kilo cat per hour
	Portal	Hepatic	
96D	9.4	10.5	875
96E	16.4	17.7	1000
102A	9.7	11.7	1940
Mean			1265 $\pm$ 410

pared and equilibrated in suitable buffers at 38° C. for a period of 2 hours. The slices and the medium were then analyzed for ketone bodies and the amount of ketones formed by the liver calculated to body weight per hour. The figure (Fig. 10) shows that the total ketone formation by the liver was greatly in excess of the preliminarily determined ketone excretion. The difference can only represent the ketone body utilization by the peripheral tissues, chiefly muscle, of the intact diabetic cat. The mean value in the case of 8 diabetic cats was 1100  $\mu$ M/kg. of body weight/hr. which is equivalent to 2.3 gm. of fat/kg./day.

The second method stands in contrast to the other methods used in which surviving liver slices or muscle minces were equi-

brated in vitro. Possible objections to such preparations were avoided by using the intact diabetic cat. First the urinary ketone excretion was measured over a preliminary period of 2 to 4 hours. Then the abdomen was opened and with as little trauma as possible samples of portal and hepatic blood were obtained and their ketone bodies determined. In all cases, the outgoing hepatic concentration was higher than the ingoing portal ketone concentration. In order to calculate the total ketone production by the liver we used a mean value for liver blood flow taken from the literature (Schmid, 14). The ketone body production by the liver so calculated was again greatly in excess of the ketone body excretion determined during the preliminary period. The difference is utilization. The results (equivalent to 2.2 gm. of fat/kg./day) are in agreement with our three types of in vitro experiments in showing a marked utilization of ketone bodies by the peripheral tissues of the diabetic cat.

On the basis of these experiments and other evidence it seemed reasonable to us to exclude the overproduction hypothesis as an explanation of the diabetic defect and adhere to the underutilization hypothesis. The diabetic in calling for reserves of energy from fat, could partially oxidize the fatty acids in the liver to ketone bodies which could be freely used in the periphery without insulin and without the necessity of coupling this oxidation with the oxidation of carbohydrates.

But there still remained two things to be done. The first was to obtain evidence for the oxidation of ketones by the peripheral tissues in human cases of diabetes mellitus, and the second was to re-examine the so-called ketogenic-antiketogenic ratio which still represented in the literature the expression of the obligatory coupling hypothesis in the human diabetic.

The assumptions used were clearly stated by Shaffer (15) as follows: "The hypothesis states that antiketogenic in the human subject is based upon a ketolytic reaction in the body between acetoacetic acid, the first formed of the acetone bodies, and a derivative of glucose (or other antiketogenic substance), the compound being further oxidized, but that failing to react with keto-

lytic substance, acetoacetic acid is resistant to oxidation, accumulated and is . . . excreted. . . . The fact that one finds at the threshold of ketosis an approximately constant ratio between the number of molecules of the precursors of acetoacetic acid and of

### Diabetes Mellitus, Bessie B.

(Wilder, Boothby, and Beeler, 1922)

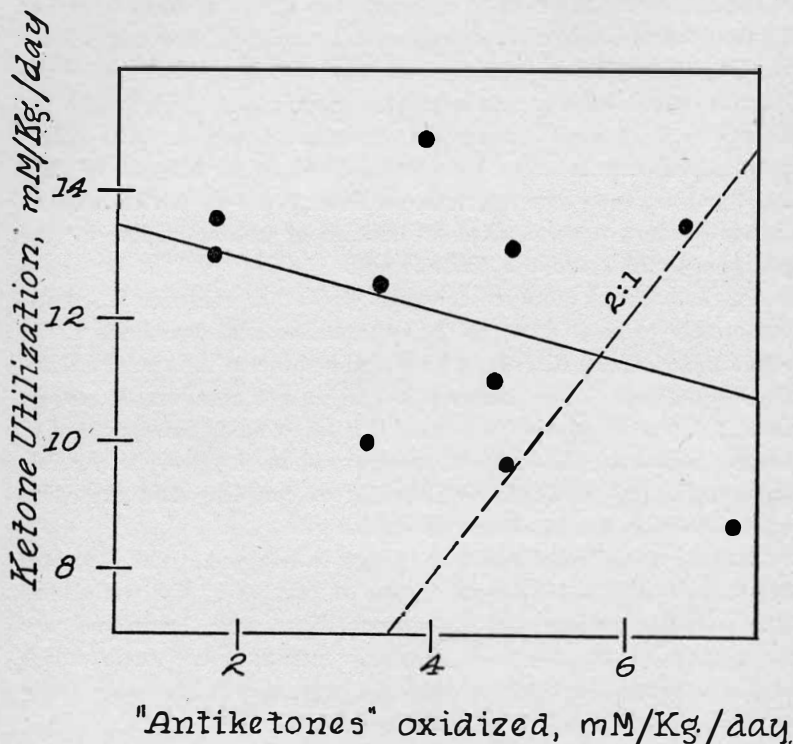


FIG. 11.

glucose in the metabolic mixture, must mean that the further oxidation of acetoacetic acid constantly taking place under normal conditions is accomplished through a chemical reaction with a derivative of glucose. . . ."

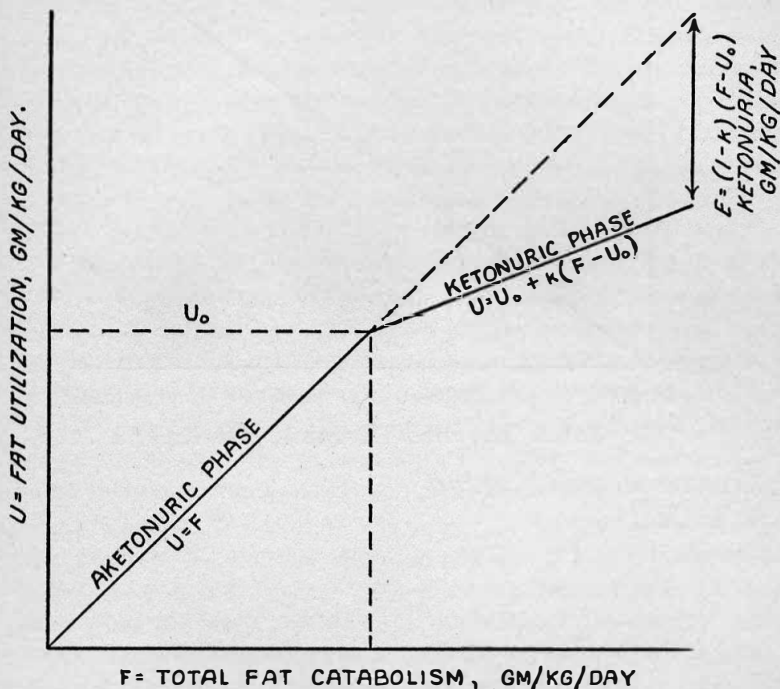
Fortunately, there is in the literature a sufficient amount of the necessary data for re-testing this hypothesis in subjects with diabetes mellitus. The cases are all classical ones, reported in the literature before the advent of insulin when marked ketonuria was, of necessity, a frequent accompaniment of the disease. Similar data on human diabetics will in all probability never be obtained again, since it is unlikely that patients with marked ketonuria will be allowed to remain untreated over long periods of time. The data include calorimetric measurements of the metabolic mixture of proteins, fats and carbohydrates together with the total ketone body excretion. Hence it is possible to calculate the total carbohydrate or so-called antiketones oxidized. In addition, from the total fat catabolized and the urinary ketone excretion, it is possible to calculate the total fat utilized or its equivalent in ketones. The best way to examine the data is to put it in the form of an equation and apply the data to the equation by statistical methods. When there is a definite excess of ketone excretion the equation is a simple one and is shown in the slide (Fig. 11) by the dotted straight line labelled 2:1. The equation merely restates the hypothesis, namely, that two molecules of ketones, but no more, are oxidized for every mole of antiketones oxidized. The figure shows the data in one case, that of Bessie B. of Wilder, Boothby and Beeler (16), one of the best studied cases of the series. The ordinate shows the mM of ketone utilization calculated from the metabolic mixture. The abscissa shows the mM of antiketones oxidized. If there were anything in the obligatory coupling hypothesis the observed points should fall on the line marked 2:1 which is calculated for a 2:1 ketogenic-antiketogenic ratio. But the true line for the data runs in the opposite direction with no relation whatever to the theory. Moreover it is important to note that the intercept constant, when antiketones were zero, is about 14 and is very greatly in excess of zero which it should be according to the theory. That is to say, Bessie B. was utilizing either ketones or fat even in the practically complete absence of carbohydrate oxidation, a conclusion in conformity with the experiments with the diabetic cats and

opposed to the obligatory coupling hypothesis. Analysis of five other cases of diabetes mellitus for which complete data were available in the literature all showed the same thing as did this illustrative case. There was found no significant correlation between antiketones and fats oxidized. It seemed to us from this analysis that the last remnant of the obligatory coupling hypothesis has vanished for there was no suggestion in the data that there was any fixed molecular ketogenic-antiketogenic ratio. On the other hand, there were strong indications that these patients, many of whom were almost complete diabetics were, like our diabetic cats, completely oxidizing ketones or fats without simultaneous oxidation of carbohydrates.

It was found possible to state a simple hypothesis of fat metabolism in diabetes mellitus which would conform to the observations in these clinical cases and with the experiments with animals. It is as follows: Up to a certain level all fat catabolized is completely oxidized; hence there is no ketonuria. Beyond this level all fat catabolized is not completely oxidized; hence part of the fat catabolized is excreted unburned in the form of ketone bodies. The action of carbohydrates is simply to spare fat oxidation; there is no molecular reaction, hence there is no molecular ratio between the two.

The diagram (Fig. 12) shows the implications of this hypothesis. The figure shows the amount of fat in the metabolic mixture. On the abscissa,  $F$  is the total fat catabolized, that is, the fat poured into the stream of intermediary metabolism and undergoing oxidations of one sort or another. On the ordinate is shown by  $U$  the total fat which is completely oxidized. Both of these values are in gm./kg./day. There are two phases in the metabolism of fat, the aketonuric and the ketonuric phases. Starting from zero and increasing the amount of fat catabolized, we pass through the aketonuric phase along the line marked  $U = F$ . Here all of the fat in the metabolic stream is completely utilized and there is no ketonuria. But, at any given state of activity, the ability of the organism to mobilize exactly that amount of fat which can be completely oxidized appears to be limited. This

upper limit is shown by the dotted line marked  $U_0$ . I call this the maximal aketonuric fat utilization. If the call for fat calories exceeds this aketonuric level, it is answered in the following way: Extra fat is catabolized above the aketonuric level, but only part of this extra fat is completely oxidized. This is shown by



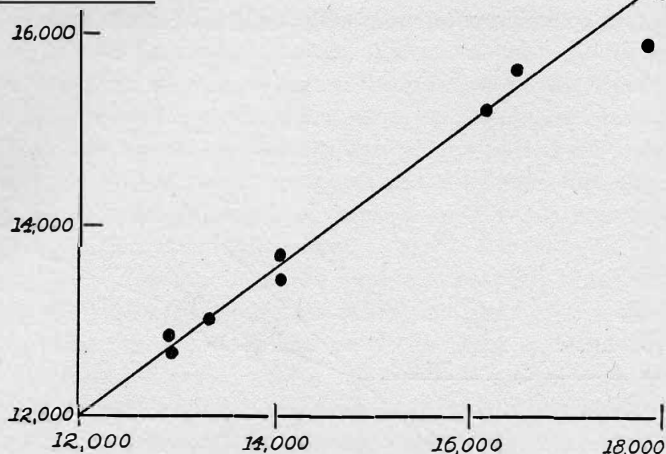
*Schema of Fat Metabolism in Diabetes Mellitus.*

FIG. 12.

the bending of the line marked ketonuric phase away from the extended dotted line which would represent complete oxidation. The difference between these two lines, indicated by the double arrow on the right, is not utilized and hence is excreted in the form of the ketone bodies. In other words, the diabetic can only

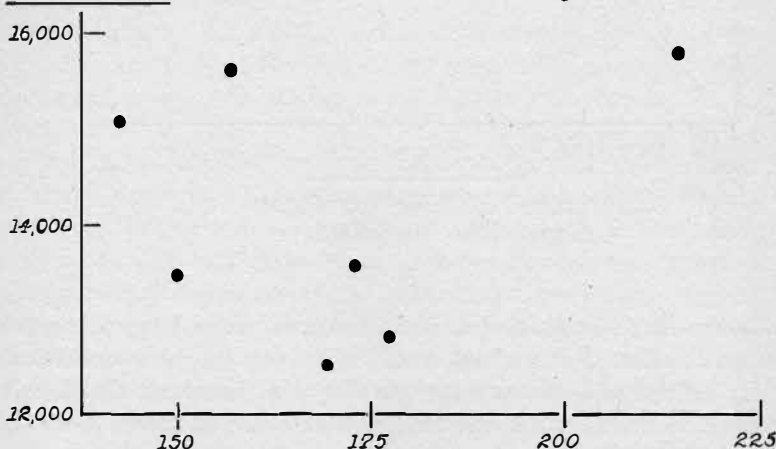
Diabetes Mellitus. No. 740. Joslin, 1915.

Ketones utilized/Kg./hr.  
Micromoles



I.- Total ketones formed, uM/Kg./hr.

Ketones utilized/Kg./hr.  
Micromoles



II.- Total "antiketones" oxidized, uM/Kg./hr.

FIG. 13.

increase his energy from fat above the basal aketonuric level by wasting part of the fat catabolized by the excretion of ketone bodies combined with base at the risk of acidosis.

This hypothesis can be tested by applying the data of diabetic cases in the ketonuric phase. There should be found, as indicated in the diagram, a straight line relation between the fats completely oxidized and the total fat catabolized. When this line is extended back to the point where there is no ketonuria, the value of the aketonuric fat utilization is obtained and this should be about the same value for all cases. In other words, if we plot the data obtained from a diabetic during ketonuria, we should obtain a line similar to the one marked "ketonuric phase" in the slide.

I show (Fig. 13) one representative case, No. 740 of Joslin (17). The data represent as before, the metabolic mixture determined by calorimetric methods. For contrast the diagram shows on the left the present hypothesis and on the right the ketogenic-antiketogenic hypothesis. On the left the total fat catabolized calculated as ketone bodies is plotted against the total fat completely utilized. The observations fall about a straight line as expected from the hypothesis. At 1200  $\mu\text{M}/\text{kg.}/\text{hr.}$  of ketone equivalent to 2.1 gm. of fat/kg./day, catabolism and utilization are equal, hence there is no ketonuria and 2.1 is the aketonuric fat utilization in this case. When ketones formed were 1800  $\mu\text{M}$  only 1600  $\mu\text{M}$  were utilized. In other words, about 200  $\mu\text{M}$  or 1/9 of the fat catabolized was excreted as ketones. In the segment on the right of the slide the data from the same case are plotted according to the ketogenic-antiketogenic hypothesis. Here, as in the cases hitherto illustrated, there is no correlation between the data and the hypothesis.

I have preferred to talk about utilization in order to emphasize the question of fat oxidation in the diabetic, but the hypothesis could just as well be framed in terms of urinary ketone excretion. By way of illustration I show the data in the case of Cyril K. of Gephart, Aub, DuBois and Lusk (18) (Fig. 14). Total fat metabolism is expressed in ketone equivalents as  $\mu\text{M}/\text{kg.}/\text{hr.}$  and



the urinary ketone in the same units. The data fall about a straight line as expected from the hypothesis. Note that when the urinary ketones are zero, fat catabolism is equivalent to 1500  $\mu\text{M}/\text{kg}./\text{hr.}$  or about 150 gm. of fat per day. This was Cyril K's basal aketonuric fat utilization value. Note further that when

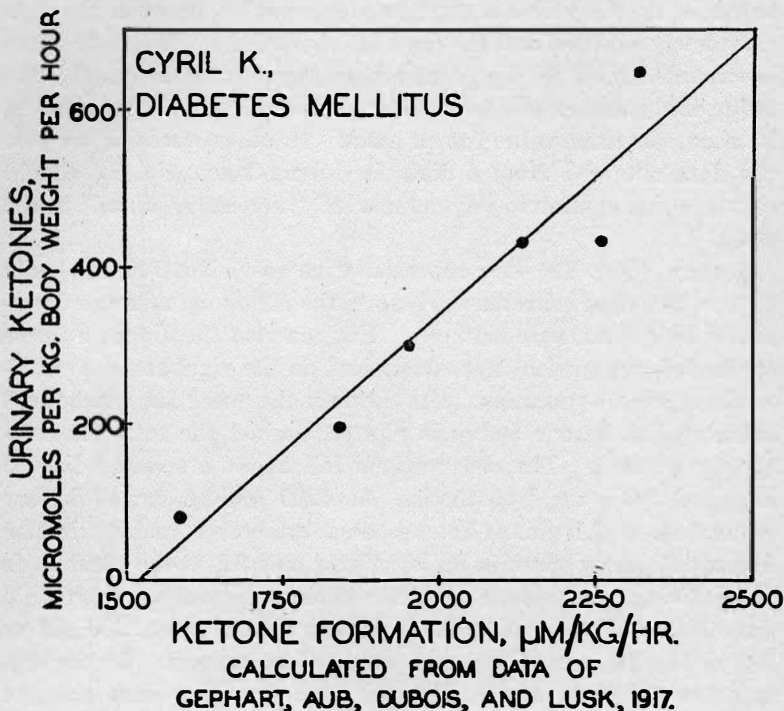


FIG. 14.

he increased his fat catabolism by 1000  $\mu\text{M}$  he excreted about 600  $\mu\text{M}$  out of this extra 1000. In other words, Cyril K. could only use about 40 per cent of the fat catabolized above his basal aketonuric fat utilization level.

All cases for which there were data available in the literature (Fig. 15) showed the same relations as those shown in these illustrative cases. The analysis of these cases is shown in this slide.

There are twelve cases. The values for the aketonuric fat utilization are shown in the last column. With the exception of Mosenthal and Lewis' (19) case E. W. which was the only case with infection and fever, these values are all concordant and the mean value is equivalent to 2.5 gm. of fat/kg./day. The findings here

FIG. 15.

*Summary of Maximal Basal Aketonuric Fat Utilization in Cases of Diabetes Mellitus*

Case	Reference	Maximal aketonuric fat utilization in equivalents of ketone bodies
<i>Group I</i>		<i>mM. per kgm. per day</i>
Cyril K. ....	Gephart, Aub, DuBois and Lusk (18)	35
Bessie B. ....	Wilder, Boothby and Beeler (16)	34
Kramer ....	Shaffer (15)	35
740 ....	Joslin (17)	28
E. W. ....	Mosenthal and Lewis (19)	(47)*
Jervis B. ....	Richardson and Ladd	23
<i>Group II</i>		
Ray H. ....	Richardson and Ladd	37
Chris. Q. ....	Richardson and Ladd	26
Harold J. ....	Richardson and Ladd	37
George H. ....	Richardson and Ladd	37
Frank B. ....	Richardson and Ladd	37
K. A. ....	McClelland, Spencer, and Falk	40
Mean (11 cases) Equivalent in grams of fat		$34 \pm 1.6$ (S. E. of mean) $2.5 \pm 0.12$

\* Excluded from basal mean on account of fever (102° F.).

outlined are offered as proof that the hypothesis of fat metabolism in the diabetic stated previously is in conformity with the quantitative experimental and clinical data available for testing it.

One more point relating to fat metabolism remains to be discussed. There are two possible types of fat metabolism. The first is the direct oxidation of fat both initiated and completed

in the peripheral tissues. Almost nothing is known about the chemical reactions or enzymes involved in this type except that it appears never to give rise to ketonemia. The second is the indirect type in which fat oxidation is initiated in the liver by the sole formation of ketones and completed in the periphery by the subsequent oxidation of these ketones. There are reasons for believing that both of these types are operative at the same time. For example, we found with liver slices from diabetic cats a maximum ketone formation equivalent to 2.3 gm. of fat per kg./day. Practically all of this was utilized. But the total basal metabolism of the diabetic cat, according to Ring and Hampel (20) is equivalent to about 8 gm. of fat/kg./day. That is to say, only 30 per cent of the total fat metabolism could be accounted for by the mechanism of indirect fat oxidation. Recently Crandall, Ivy and Ehni (21) using London cannulae, came to the same conclusion in the case of normal fasting dogs. They could only account for about 50 per cent of the total fat metabolism by hepatic ketogenesis. A second reason for believing in the dual mechanism of fat oxidation is found in a consideration of the oxygen requirements of the liver. If all of fat metabolism went through preliminary ketone formation by the liver, the amounts of oxygen required would be very greatly in excess of the values which are actually found for the oxygen consumption of that organ. Thirdly, diabetic muscle, when equilibrated in vitro, shows a respiratory quotient of 0.7, indicating an ability on the part of the muscle to oxidize fats directly.

The results of our experiments together with related evidence in the literature may be summed up as follows:

The diabetic who by reason of insulin lack is unable to utilize carbohydrates to the full measure of his metabolic needs, must fall back upon fat for his energy requirements. Part of this need is met by the complete oxidation of fat in the muscles themselves. However, a considerable fraction, estimated as  $\frac{1}{3}$  to  $\frac{1}{2}$  of the total caloric needs from fat, is obtained by a preliminary oxidation of fats in the liver to ketone bodies only. Neither acetic acid nor glucose are formed by this oxidation. These hepatic

ketone bodies are freely utilized for energy by the periphery without insulin and without the necessity of simultaneous carbohydrate oxidation. This reserve mechanism, however, appears to be incapable of fine regulation so that when the demand for fat calories exceeds a certain level, approximately 2.5 gm. of fat/kg./day for the resting state, ketone bodies in excess of needs are formed by the liver. The excess is excreted in the urine. If this excessive fat catabolism continues unchecked ketosis and coma follow.

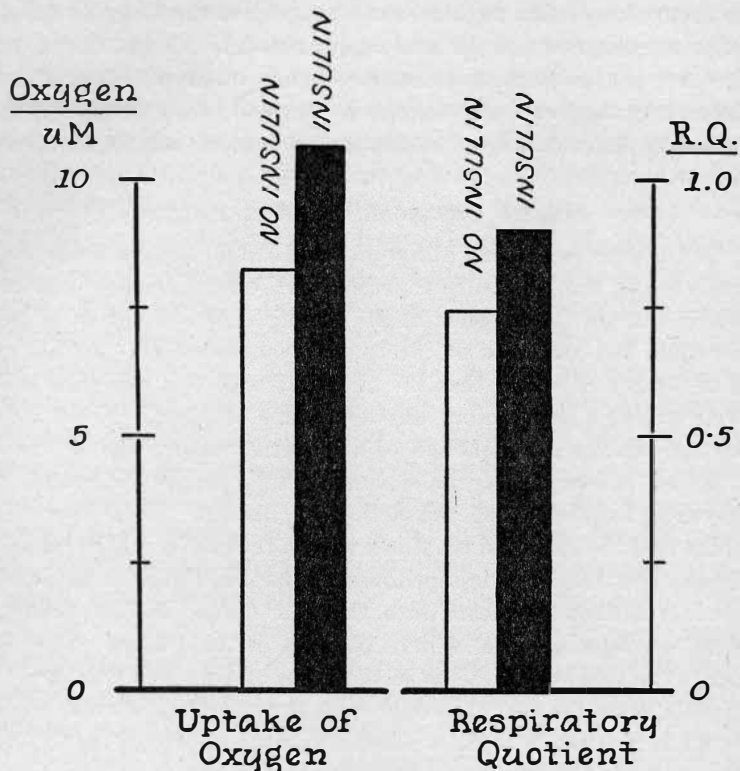
#### SPECIFIC CHEMICAL ACTION OF INSULIN

In the time remaining at my disposal I shall discuss some of the evidence in the literature together with experiments of our own which bear on the problem of the specific chemical action of insulin upon the processes of intermediary metabolism. Initially it is necessary to admit that the summation of this evidence is far from giving a satisfactory answer to this problem. Nevertheless certain conclusions although of a tentative nature can be drawn.

First, however, may be discussed what might be called the physical hypothesis of the action of insulin. This hypothesis holds that the chemical reactions within the cell by which carbohydrate is oxidized are independent of insulin, but that ordinarily glucose cannot permeate into the cell unless insulin produces some surface change which permits it to do so. The evidence for this hypothesis is in the main of a negative nature, i.e., it rests upon the fact that only with the greatest difficulty or not at all is it possible to demonstrate *in vitro* effects of insulin and then only in the presence of cellular structure.

The demonstration that insulin does produce changes in the metabolism in cell-free systems would rule out this hypothesis. We have accomplished this demonstration in the case of sand-homogenized extracts of pigeon or rat muscle equilibrated *in vitro* under suitable conditions. The precise conditions for the constant reproducibility of this effect are not clear, but in two thirds of 80 experiments there was found a significant increase of total oxygen uptake and in about one half of 62 experiments there was an increase of the respiratory quotient in the presence of added

insulin indicating an augmentation of carbohydrate metabolism by the extract. One protocol of the positive results of the series is shown (Fig. 16). On the left the figure shows the oxygen up-



*Exp. no. 69 - Action of Insulin upon Rat Muscle Extract.*

FIG. 16.

take of 2 cc. of aqueous rat muscle extract without and with insulin at  $1 \mu/\text{cc}$ . In the presence of the insulin there was found a marked increase in oxygen uptake in 80 minutes. On the right are shown the respiratory quotients of the samples. With insulin there is an appreciable increase.

In this connection mention must also be made of experiments by Banga, Ochoa and Peters (22). They found with brain dispersion and with pyruvate as a substrate that insulin in vitro not only increased the oxygen consumption but also rendered more complete the oxidation of pyruvic acid.

On the basis of these experiments the exclusion of the physical hypothesis seems reasonable and studies of possible catalytic chemical mechanisms of insulin action appear in order. In the main, discussion has centered about two possibilities: (1) Action upon glycogen synthesis in liver and muscles, and (2) action upon carbohydrate oxidation chiefly in muscles.

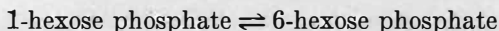
Consider first the question of glycogen formation. Experiments with intact animals have told us little about the mechanism of insulin action in this reaction. Indeed, statements are made in the literature that, in the liver, glycogen synthesis is essentially independent of insulin. There are, for example, the experiments of Einar Lundsgaard (23) who found, following perfusion with fructose enriched blood, just as great a deposition of glycogen in *isolated livers from depancreatized cats as from normal cats.*

Experiments in vitro have, in the main, been equally unilluminating. We have been unable, using liver slices from normal and diabetic cats or normal young rabbits, to influence the glycogen formation by the addition of insulin to the medium. Such has been the experience of others, in particular, Ostern and Holmes (24) using normal rabbits. On the other hand, there are reasons for believing that insulin does have an effect upon hepatic glycogen formation. For example, Banting (25) found that depancreatized animals rapidly increase their liver glycogen concentrations following injection of insulin. We have found in depancreatized cats intensively treated with insulin over the relatively short period of 2 to 4 hours increases of hepatic glycogen over initial values. In this connection must be mentioned the experiments of Taubenhaus, Levine and Soskin (26). Using normal rats previously injected with insulin, they found that liver breis equilibrated with glucose showed less glycogen breakdown than did the controls. They concluded that insulin has a direct effect upon hepatic glycogenolysis.

In muscle, glycogen formation appears to be partially independent of insulin action as indicated by experiments of the type presented by Long, Lukens and Fry (27) who found glycogen formation from glucose in depancreatized cats although at rates less than normal. But in contrast to the case of the liver, there is ample evidence that insulin markedly accelerates this reaction. It will suffice to mention the recent significant experiments of Gemmill (28). He found, using diaphragms from normal rats *in vitro*, a significantly greater synthesis of glycogen from glucose upon addition of insulin to the medium. This observation has been confirmed by Hechter, Levine and Soskin (29) and by Stadie and Zapp (unpublished experiments).

But by what chemical mechanism does insulin produce these effects upon glycogen formation in liver or muscle? Hypotheses, of course, must be formulated in terms of the current conception of glycogen formation from glucose. According to the work of Cori, Parnas and others two main steps are involved: (1) A preliminary phosphorylation of glucose to 6-hexose phosphate, and (2) a further transformation of 6-hexose phosphate through 1-hexose phosphate to glycogen. The first step of phosphorylation is strictly oxidative and requires the input of energy which must come from the oxidation of specific types of substrates. The efficiency of phosphorylation, i.e., the amount of hexose phosphate and hence potential glycogen formed per mole of substrate oxidized, is known to vary. The recent experiments of Colowick, Kalekar and Cori (30) illustrate this. For example, at top efficiency, according to a calculation of Lipmann (31), only one mole of glucose need be sacrificed to bring about the formation of 12 to 24 glycogen equivalents. Since phosphorylation of hexose appears to be brought about by the oxidation of carbohydrate intermediates only, it is possible to propose the hypothesis that insulin, by catalyzing such oxidations, would increase the efficiency of phosphorylation and hence glycogen formation. Such a unitary hypothesis would explain by one common chemical mechanism how insulin promotes carbohydrate oxidation and also regulates glycogen formation. At present there is not direct evidence for it.

The second general step in glycogen formation, i.e., the transformation of 6-hexose phosphate through 1-hexose phosphate to glycogen is non-oxidative and as Cori, Colowick and Cori (32) have shown is not influenced by insulin at least when studied in purified enzyme solutions. The effect of insulin in inhibiting formation of 6-hexose phosphate from glycogen reported by Gill and Lehmann (33) is attributed by them to a non-specific protein reaction with magnesium, a necessary component of the system. Nevertheless, the suggestion has been made by Taubenhaus, Levine and Soskin (26) that it is the reversible non-oxidative reaction:



which is influenced by insulin. They propose this hypothesis on the basis of the experiments already quoted in which liver breis from insulin injected normal dogs equilibrated aerobically with added glucose and insulin show diminished formation of free glucose from glycogen.

Apparently these two hypotheses of the chemical action of insulin in glycogen formation or breakdown, namely, on the oxidative phosphorylation of hexose, or on the non-oxidative transformation of hexose phosphate are the only ones seriously considered. At present no decision can be made between them.

#### ACTION OF INSULIN UPON OXIDATIVE PROCESSES

The problem of the specific chemical action of insulin upon oxidative processes is likewise obscure. I have discussed reasons for believing that insulin plays no direct role in the oxidation of fats. There is little evidence that it affects directly the metabolism of protein although this view has been advocated notably by Bach and Holmes (34) who concluded from experiments in vitro with rat liver slices that insulin inhibits the deamination of amino acids in the liver and thereby decreases glyconeogenesis from protein. Our own experiments (Stadie, Zapp and Lukens (1)) offer a much different interpretation of this observation. Mirsky (35) offers a still different effect of insulin upon protein metabolism, namely, that insulin promotes the synthesis of pro-



tein in muscle from amino acids. But if the possible conversion of fat to carbohydrate is ruled out, which appears to be the case, it is difficult if not impossible to integrate the large amount of experimental data on the effect of insulin in the intact normal or diabetic animal except by assuming that insulin has catalytic effects upon the oxidation of carbohydrate.

As representative of the type of experiment which indicates that insulin increases the oxidation by muscle of carbohydrate may be mentioned the work of Cori and Cori (36). They found in a series of intact rats that insulin doubled the rate of carbohydrate oxidation as compared to controls. These experiments require the calculation of metabolic data from respiratory metabolism. For this reason they have been criticized (Soskin, 37) but more direct evidence is available. For example, Shorr (38) showed that the isolated muscle from diabetic dogs had a respiratory quotient of 0.7, that of fat, which did not increase upon the addition of glucose or lactic acid, in contrast to the behavior of isolated muscle from normal dogs. Cruickshank (39) showed that the heart from diabetic dogs perfused with glucose containing blood increased its respiratory quotient from 0.7 upward only upon the addition of insulin to the perfusion fluid. Recently we have shown (Stadie, Zapp and Lukens, unpublished experiments), using depancreatized cats, that muscle strips taken initially have low respiratory quotients when studied *in vitro*. Upon subsequent intensive treatment of the cat with insulin over a period of 2 to 4 hours, the isolated muscle increased its respiratory quotient toward 1 indicating a restoration of carbohydrate metabolism.

In spite of this apparent wealth of general evidence, information concerning the specific chemical mechanisms of the catalysis of carbohydrate oxidation by insulin is meager indeed. This is hardly surprising in view of the fact that knowledge of the oxidative processes of carbohydrate metabolism is at present incomplete and confusing. In general, carbohydrate metabolism in muscle involves (1) a glycolytic cycle leading to the formation from glycogen of lactic acid through pyruvic acid, and (2) an

oxidative cycle leading to the oxidation to  $\text{CO}_2$  and water of the intermediates or end products of the glycolytic cycle.

With respect to the glycolytic cycle the present evidence indicates that it is independent of insulin. On the other hand, with respect to the oxidative cycle there is one which has been proposed by Krebs and Eggleston (40) in which insulin is supposed to play a role. Briefly the scheme is as follows (Fig. 17).

#### KREBS' CITRIC ACID OXIDATIVE CYCLE

1.  $\frac{1}{2}$  Glucose (triose) + Fumaric acid +  $2 \text{ O}_2 = \text{Citric acid} + \text{CO}_2 + \text{H}_2\text{O}$
  2. Citric acid +  $\frac{1}{2} \text{ O}_2 = \alpha$  ketoglutaric acid +  $\text{CO}_2 + \text{H}_2\text{O}$
  3.  $\alpha$  ketoglutaric acid +  $\frac{1}{2} \text{ O}_2 = \text{Succinic acid} + \text{CO}_2 + \text{H}_2\text{O}$
  4. Succinic acid +  $\frac{1}{2} \text{ O}_2 = \text{Fumaric acid}$
- Summation:  $\frac{1}{2}$  Glucose +  $3 \text{ O}_2 = 3 \text{ CO}_2 + 3 \text{ H}_2\text{O}$

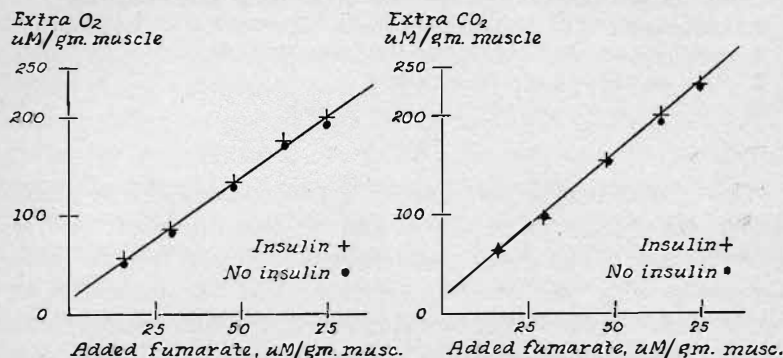
FIG. 17.

The  $\text{C}_4$  dicarboxylic acids, succinic, fumaric, malic, and oxaloacetic, are present in all tissues and play an important role in intermediary metabolism. According to Krebs, fumaric acid condenses upon oxidation with glucose with the formation of citric acid. Upon further oxidation of the citric acid,  $\alpha$  ketoglutaric, succinic, and finally fumaric acids are formed thus completing the cycle. Each step is accompanied by oxygen uptake and in the first three there is  $\text{CO}_2$  elimination. The final result is the oxidation of glucose to  $\text{CO}_2$  and water. Krebs first showed with pigeon muscle in vitro that insulin increased the oxygen uptake in the presence of citric acid or the intermediate  $\text{C}_4$  acids. In general, this observation has been abundantly confirmed by Shorr and Barker (41), by Stare and Baumann (42) and by Stadie, Zapp and Lukens (2). This increase of  $\text{O}_2$  uptake has been interpreted by Krebs to mean that insulin, either by itself or in association with the enzymes of the muscle has catalyzed some step in the cycle.

This discovery of Krebs was indeed significant and aroused hopes that a biological system had been found which would permit the chemical dissection of the insulin effect upon carbohydrate intermediary metabolism. But with further experiment its meaning has become obscure. The citric acid cycle itself has not

escaped criticism (see, for example, Stare, Lipton and Goldinger, 43). Further, the effect appears confined to pigeon muscle for Shorr and Barker (41) were unable to find it using muscle from chickens, cats, dogs, or rabbits. Stadie, Zapp and Lukens (2) found the effect independent of the presence of added citrate and they did not find an enhanced effect in the case of depancreatized pigeons. With muscle mince from depancreatized cats they could find no effect whatever.

But whatever its significance the insulin effect with pigeon



Respiration of depancreatized (7 days) pigeon breast muscle mince in presence of 0.025 M malonate.

FIG. 18.

muscle mince is unquestionably real and we have experimented further with it (unpublished experiments).

In the presence of sodium malonate the reactions of the Krebs cycle are blocked at the succinic acid stage and the individual reactions of the cycle should be more or less isolated. We have been unable by in vitro experiments with pigeon muscle mince to show that any of the individual reactions so isolated are influenced by the presence of insulin in the medium. One illustrative protocol will suffice. The figure (Fig. 18) shows the total oxygen uptake and the CO<sub>2</sub> output in relation to the amount of fumarate added to the medium containing muscle mince. Included in the diagram are observations without and with insulin.

FIG. 19.

*Ketone Formation by Liver Slices from Houssay Cat*

Cat No.	Hypophy- sectomy	Pancrea- tectomy	Experi- ment	Cat weight	Liver weight	Blood sugar	Liver glycogen	Liver ketone formation per kilo cat per hr.
	1939	1939	1939	kg.	gm.	mg. per cent	per cent	micro- moles
105A	June 8	June 14	June 19	3.4	50	60	0.04	68
105B	Sept. 7	Sept. 18	Sept. 20	2.1	49	.....	0.02	104

There is no difference between the two sets of data. In other words, if, as Krebs states, insulin is a catalyst in any of the intermediate steps of the citric acid cycle the method of isolation of these steps which we have used has failed to localize the insulin action.

Brief mention might be made at this point of another hypothesis which, since its theoretical development by Van Noorden, Falta, and others of the Viennese School, has assumed various guises in the literature. It might loosely be described under the term "hormonal antagonism" or "hormonal balance." The recent classical work of Houssay has re-emphasized it. According to this, certain hormones act antagonistically or in a balancing fashion toward each other in respect to the action of each upon the metabolism of the cell. For example, in a depancreatized animal the resultant pituitary excess is perhaps as significant as the lack of insulin. Removal of this excess by hypophysectomy restores the metabolism toward a normal state as in the Houssay animal.

It would be useless in our present state of knowledge to attempt to frame this hypothesis in terms of specific chemical mechanisms. Shorr and his colleagues (44) in the light of their experiments have framed it in general terms. They found that the metabolism in vitro of cardiac muscle from depancreatized dogs was initially of the diabetic type but upon equilibration for several hours became normal in its ability to oxidize carbohydrate. They suppose a primitive cellular metabolism upon which is superimposed hormonal effects. Because of lack of insulin the cardiac muscle shows an unbalanced type of metabolism which is called diabetic. Within a few hours in vitro, however, unbalancing factors are removed or destroyed and the metabolism becomes that of the primitive state which in many respects resembles normal.

In this connection experiments from our own laboratory might be cited. We found (Fig. 19) that liver slices from depancreatized-hypophysectomized cats when equilibrated in vitro, in contrast to livers from depancreatized cats, produced almost no

ketones. In fact, the ketone formation was even less than that formed by liver slices from fasted normal cats. The table shows a mean ketone formation of about  $90 \mu\text{M/kg. body weight/hour}$  which is about  $\frac{1}{3}$  of the value for normal fasted cats and about 8 per cent of the mean value in a series of depancreatized cats. In other respects the hepatic metabolism appeared normal and could be said to be restored to a primitive condition. But the removal of the anterior pituitary has eliminated a factor which enabled the liver to oxidize fats to ketone bodies. Whether this factor is a specific fat oxidation enzyme originating in the pituitary is entirely a matter of conjecture. But the metabolism in this primitive state is less efficient because the ability of the liver to supply a considerable fraction of the total metabolic needs from fat by preliminary partial oxidation to ketones has been lost and the muscles must fall back almost entirely upon the mechanism of direct oxidation to supply their fat calories. A reflection of this inefficiency is perhaps found in the experiments of Lee and Ayres (45) who found that hypophysectomized rats as contrasted to normals were relatively limited in their ability to mobilize and use stored body fat and consequently were forced to draw upon body protein to supply energy demands.

The search for the locus of the chemical action of insulin still continues in the laboratories of those devoted to the subject. But one is forced to admit that a survey of twenty years of literature since the discovery of insulin does not reveal conclusive results. The chemical mechanism of insulin action eludes definition and at present there is no unequivocal evidence which enables us to name with certainty any reaction which it mediates, accelerates or inhibits. My feeling is that insulin is a hormonal catalyst, that its action is chemical upon the oxidative processes of the intermediary metabolism of carbohydrates, and that eventually its many physiological actions will be explained upon the basis of a single general type of chemical reaction. At all events, the problem of the chemical action of insulin is one of the most interesting unsolved problems in the field of diabetes mellitus.

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