Effect of Some Oral Hypoglycemic Agents on Hepatic Protein Synthesis

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EFFECT OF SOME
ORAL HYPOGLYCEMIC AGENTS
ON HEPATIC PROTEIN SYNTHESIS

by

LAWRENCE R. DECHATELET

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science

June
1965
LIFE

Lawrence Robert DeChatelet was born in Chicago, Illinois on December 24, 1940. In June 1959, he graduated from Saint Ignatius High School, Chicago, Illinois and then attended Loyola University, Chicago, Illinois, from where he received the Degree of Bachelor of Science in Chemistry in June, 1963.

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On December 26, 1964, the author married the former Miss Mary Patricia Dolan of Chicago, Illinois.
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ABSTRACT

The action of the hypoglycemic compounds, tolbutamide and phenethylbiguanide, on protein biosynthesis was investigated by the use of rat liver homogenates, the necessary cofactors and leucine-$^{14}C$. The results show that the incorporation of leucine-$^{14}C$ into hepatic protein is inhibited by both compounds, though in a strikingly different manner. The inhibition by tolbutamide is linear with respect to concentration whereas the inhibition by phenethylbiguanide is not evident until a certain concentration is reached. At this point, the amount of incorporation decreases markedly until it levels off at a very low value. It has been postulated that there is some substance present which combines with phenethylbiguanide and renders it inactive toward inhibition of protein synthesis. Glucose was investigated in this respect and found to have no effect on either tolbutamide or phenethylbiguanide induced inhibition.

The catabolism of leucine was measured by the amount of $^{14}CO_2$ liberated in the course of the incubation. Both tolbutamide and phenethylbiguanide were found to inhibit $CO_2$ production. Again, inhibition by tolbutamide was linear with respect to concentration. Phenethylbiguanide caused a pronounced initial inhibition which tended to level off near the same concentration where
the protein inhibition first occurs. This suggests that one of the reasons that low concentrations of PEBG does not inhibit protein incorporation is due to the great decrease in leucine catabolism. Since less leucine is broken down (catabolized), there is necessarily more present to be incorporated into the protein. According to this postulation, the initial great decrease in leucine catabolism compensates for the inhibition in protein anabolism. Once the inhibition of protein catabolism has levelled off, the inhibition of incorporation by added PEBG becomes evident.

The results found are exactly opposite those reported by Krahl (14) for insulin. Whereas tolbutamide and phenethylbiguanide inhibit protein synthesis in vitro, insulin accelerates it. Moreover, while the accelerating action of insulin is dependent on the presence of glucose in the medium; the inhibitory action of the oral hypoglycemic agents is totally unresponsive to the addition of glucose.

These results suggest that the oral hypoglycemic agents studied, although very proficient at lowering blood sugar, do not possess all the other attributes of insulin. They cannot aid the negative protein balance associated with diabetes; they may, in fact, due to their inhibitory effects, aggravate the condition.
CHAPTER I

DIABETES AND THE ACTION OF INSULIN

History

A reference in the ancient Papyrus Ebers (105) to a condition that may have been diabetes mellitus is indefinite, but it does suggest that a written record of the disease may be dated as far back as 1500 B.C. Whether or not this is true, a good clinical description was given by Celsus (30 B.C. to 50 A.D.). It was however, another Roman, Aretaeus of Cappadocia (30 A.D. to 90 A.D.) who coined the term "diabetes" meaning literally "to pass through". The name derives from the fact that it was considered to be a disease of the kidney because of the polyuria.

The glycosuria associated with diabetes is not mentioned in the European literature on the subject until the seventeenth century, but it was described very completely by the Arab physician Avicenna about the year 1000 A.D. Even before this time, Chinese, Japanese and Hindu writings indicate that a disease associated with a sweet urine was known to these people.

Thomas Willis (142) was the first European to recognize the glycosuria associated with diabetes. This opened the door to innumerable observations on the subject. Cawley in 1788 was ap-
parently the first to associate diabetes with the pancreas. He found multiple caliculi and destruction of pancreatic tissue at the autopsy of a patient who had died of diabetes (140).

In the nineteenth century, the contributions of Claude Bernard in the field of carbohydrate metabolism stand out. He established the presence of glycogen in the liver and propounded the theory of sugar formation from glycogen. He failed to recognize the role that the pancreas plays in controlling carbohydrate metabolism, but he did theorize that the high blood sugar levels found in diabetes were due to an overproduction of sugar by the liver (11).

In 1869, Paul Langerhans discovered the islets in pancreatic tissue that were named in his honor by Laguesse. Langerhans did not appreciate the physiological significance of the islets and considered them to be lymph glands (77). In 1889, von Mering and Minkowski showed that totally depancreatized dogs developed hyperglycemia, glycosuria, and finally died in ketosis and coma (98). This was the first definite experimental proof that diabetes may be of pancreatic origin. Attempts by Minkowski to prepare a pancreatic extract which would have a hypoglycemic activity failed. This major advancement was due to the work of Banting and Best (8,9,10). Using pancreatic extracts made by procedures designed to prevent the action of proteolytic enzymes, i.e., utilizing cold acid and alcohol, they demonstrated an
extensive lowering of blood and urine sugar in seventy-five consecutive tests in ten different depancreatized dogs. The first crystalline insulin was prepared by Abel of Johns Hopkins in 1926, (1). Mention must be made here of the monumental chemistry performed by Sanger and his co-workers in elucidating the structure of insulin (117).

Research along other lines continued unabated. Houssay established the hypersensitivity of hypophysectomized toads and dogs to insulin and proved for the first time that experimental diabetes produced by total extirpation of the pancreas can be ameliorated by hypophysectomy (59). The first practical oral hypoglycemic agent, 1-butyl 3-sulfonylurea (also called carbutamide) was reported in 1955 by Franke, Achelis Betram et al. (2,14,45). Although it was soon found to be toxic and was withdrawn from clinical use in the United States, it is the parent compound which led to the discovery of the highly successful arylsulfonylureas.

Recent research has been directed toward elucidating the mechanism by which insulin acts. So much work has been performed on this phase that it is impossible to enumerate individual investigators.

CARBOHYDRATE METABOLISM

The primary metabolic defect in diabetes is a failure or
aberration in the metabolism of carbohydrate. This is obvious from the high blood glucose concentration. There can be no doubt that insulin is concerned with an early stage of the metabolism of glucose, but whether or not this is its sole action, and the mechanism by which it is achieved, are still matters for discussion and experimentation. The first reaction of glucose in mammalian tissue and one necessary for its further metabolism is the formation of glucose-6-phosphate through the action of glucose-kinase. This reaction has long been regarded as a site of hormonal regulation, including acceleration by insulin. There are two ways in which an acceleration by insulin can be accomplished: (1) by increasing the rate of entry of glucose into the cell through the cell membrane and (2) by increasing the rate of formation of glucose-6-phosphate by direct action on the enzyme hexokinase.

The first hypothesis as propounded by Levine (80) is based on the observation that insulin greatly enhances the uptake of glucose by extrahepatic tissues at all levels of blood glucose. This was tested using galactose, which will enter cells, but is not metabolized to any extent except in the liver, kidney and intestine. Levine and Goldstein (79) surgically removed the liver, kidney and intestine from a dog. A test dose of galactose was injected and the blood concentration determined. After this, insulin was given and it was observed that the blood galactose
level dropped appreciably. This was taken to indicate that the insulin permitted the galactose to leave the extra cellular compartment, penetrate the cell wall and enter the intracellular compartment.

The second hypothesis has no direct evidence to support it, but it cannot be overlooked as a possibility (140). It is mentioned here primarily for the sake of completeness.

LIPID METABOLISM:

In the condition of diabetes mellitus there are present four principal abnormalities in lipid metabolism.

1. blockage in fatty acid synthesis
2. increase in fatty acid oxidation
3. development of ketosis
4. increase in cholesterol synthesis

Each of these topics will be discussed briefly in turn.

I. Lipogenesis

The first convincing evidence that the diabetic possessed an impaired ability to synthesize fat was obtained by Stetten and Boxer (127) when they demonstrated that the incorporation of deuterium-labeled water into fatty acids was greatly de-
pressed in the intact diabetic rat. This finding was readily confirmed with C\textsuperscript{14} labelled glucose both in isolated liver slices of alloxan diabetic rats and in intact animals (26).

The site of this defect in lipogenesis in the metabolic scheme is difficult to locate. The reduction could be due to metabolic blocks at any of numerous biochemical steps involved in glycolysis and lipogenesis. In fact, the possible site of insulin action at the entrance of glucose into the cell or in its conversion to glucose-6-phosphate should of itself result in a decrease in fat synthesis from glucose. It should be mentioned that the diabetic block in lipogenesis has been shown to be much more severe than the block in glucose oxidation, a finding which suggests that the latter defect is not the direct and only cause of the inability to convert glucose to fatty acid. The studies of Chernick and Chaikoff (30) have demonstrated that in diabetic liver the synthesis of fat from fructose -C\textsuperscript{14}, as well as from glucose-C\textsuperscript{14}, is greatly depressed.

The consequences of this disturbance have been most dramatically shown by the experiment of Drury (37) in which diabetic rats or dogs subjected to alternate days of fasting and feeding suffered marked weight loss in the course of only one week of such treatment. Normal animals maintained their body weights on this program; likewise, diabetic rats when fed daily did not lose weight. It seems apparent that the inability of these animals to
store carbohydrate, even for a period of one day is a direct result of their inability to synthesize fatty acids, and it is evident that under these circumstances lipogenesis is a function without which the diabetic animal cannot survive.

II. Fatty Acid Oxidation

For a long time there has been suggestive evidence that in the diabetic, the rate of fatty acid oxidation is increased above normal. The total amount of carbon dioxide produced by the diabetic as measured by the basal metabolic rate tends to be normal or even increased (140).

It is evident that, since glucose utilization is reduced an increased oxidation of fat and to a lesser extent of protein must take place to yield this carbon dioxide and the necessary metabolic energy. More direct evidence for this increased rate of breakdown of fat in diabetes has been obtained by Lossow and co-workers (85). By observing the metabolism of C¹⁴- labeled tri-palmitin in diabetic and normal rats, he demonstrated that the diabetic oxidizes fatty acids to carbon dioxide at a rate considerably above the normal. Further, this increased oxidation of fat seems clearly to be due to the decreased amount of glucose undergoing oxidation, since the injection of glucose in normal animals, or the administration of insulin to the diabetic results in a
prompt depression of fatty acid oxidation.

III. Diabetic Ketosis

The factors leading to diabetic ketosis may be summarized as follows. The inability of the diabetic to utilize adequate amounts of glucose makes it necessary to rely on increased fat oxidation for energy. Fat can provide ample amounts of acetyl CoA but can supply neither pyruvate nor NADPH. As a result, the Krebs cycle is unable to maintain its integrity, and acetyl CoA can be oxidized to carbon dioxide only after the concentration of this compound is sufficiently high \((54) (46)\). The lack of NADPH prevents this excess acetyl CoA from being converted into fatty acids, as would normally occur under these circumstances, and the accumulated acetyl CoA is thereby shunted into HMG CoA and hence into the synthesis of ketone bodies.

IV. Cholesterol Synthesis

Liebow and Hellerstein \((81)\) have compiled data from literature on diabetes. It shows conclusively that a definite increase in severity and incidence of atherosclerosis accompanies the disease. It has been observed by Castiglioni \((25)\) that hypercholesterolemia very frequently accompanies diabetes. Finally, there has been a great deal of work done implicating atherosclero-
sis with blood cholesterol levels. Early studies by Windaus (143) and Schonheimer (120) indicated that in atherosclerotic aortas, the cholesterol content was greater than in normal ones. A wealth of statistical evidence relating atherosclerosis to cholesterol has been summed up by Rosenthal (113).

It has been directly shown in vitro that the livers of diabetic rats can synthesize cholesterol at increased rates. This was demonstrated by Hotta and Chaikoff (58) by measuring cholesterol after incubation of the liver slice with acetate-\textsuperscript{14}C. The mechanism of this increase is not clear at the present time.

Thus, two major medical problems atherosclerosis and diabetes, currently confronting the scientific world are seen to be intimately related. The mass of evidence demonstrating a marked incidence and severity of atherosclerosis in diabetes is incontrovertible. Provided they have the disease long enough, diabetics will almost invariably be atherosclerotic. Warren (133) has found only four patients free of atherosclerosis among 484 autopsied cases with diabetes for five years or longer. This universality of atherosclerosis in diabetes is true for young or old, male or female. Joslin (69) has stressed that the frequency and severity of atherosclerosis in diabetes is correlated with long standing uncontrolled glycosuria and hyperglycemia, with concomitant hyperlipemia and hypercholesterolemia.
PROTEIN METABOLISM

I. Effects of insulin on protein metabolism in intact animals:

There is a wide range of conflicting reports in the literature concerning the effects of insulin on the growth of animals. Some of the conclusions are invalid because they were performed on fasting animals. There seems to be complete agreement that the administration of insulin does not cause demonstrable protein anabolism in the fasting animal (15, 47, 50, 125). There is conclusive evidence that in the non-fasting animal, or in man, insulin tends to cause protein anabolism as indicated by a positive nitrogen balance (67, 71, 90, 125). However, this tendency of insulin to produce a positive nitrogen balance has not led to growth in normal animals (41, 62, 63). The principal experimental results of the influence of insulin on protein metabolism may be summarized in Table I, modified from Lukens (88).

II. Effects of insulin on protein metabolism in isolated tissues:

A. Muscle: It has been demonstrated by Krahl (73) and Sinex et al. (123) that the incorporation of \(^{14}\)C-glycine or \(^{14}\)C-alanine into protein of diaphragms from fed rats was enhanced by insulin in the absence of glucose in the incubation medium. The order of magnitude of the stimulation was as great as 40%. This appears to indicate that the stimulation of amino acid incorporation by insulin into muscle occurs by a mechanism other than the stimula-
TABLE I

STUDIES OF THE INFLUENCE OF INSULIN ON PROTEIN METABOLISM

I. Pancreatectomy results in an increased catabolism of endogenous protein as evidenced by an increased nitrogen excretion during fasting (97).

Insulin treatment of the depancreatectomized animal, or of the severely diabetic patient prevents this acceleration of protein catabolism (7,27,40,78,125).

From in vivo studies on the disposal of N\textsubscript{15} - glycine, there is some evidence that protein synthesis is defective and protein breakdown excessive in the diabetic animal (57).

II. Insulin given to normal animals:

A. Investigators who failed to observe protein anabolism:

1. No increased growth of rabbits (84).

2. No effect on fasting nitrogen balance in rats (15).

3. No effect on growth or nitrogen excretion of normal rats with or without control of diet (50).

4. No change in nitrogen excretion (65).

5. No effect on growth of rats (41,62,63).

B. Investigators who concluded that insulin promotes protein anabolism:

1. Glucose and insulin spare more protein than glucose alone in normal subjects (67).

2. Insulin causes positive nitrogen balance in fed normal dogs (125).

3. Insulin causes nitrogen retention in normal rats, with or without constant diet (90).

4. There is seen a positive nitrogen balance in patients given insulin (71).

5. Liver and diaphragm incorporate more labeled amino acid under influence of insulin if glucose is present (72).
B. Liver: The work on amino acid incorporation in liver slices has been performed by Krahl (14). He reported that the capacity of liver slices to incorporate glycine-C^{14} into glutathione or protein was impaired in liver slices from alloxan-diabetic rats, the degree of the defect increasing in proportion to the duration and severity of the diabetes. No direct in vitro effect of insulin could be demonstrated with slices from the most severely diabetic animals, although restoration of normal incorporation could be achieved by the injection of insulin into the animal ten hours or more before the start of the experiment.

The situation with slices from mildly diabetic rats was quite different: insulin added in vitro raised the incorporation of glycine-C^{14} into glutathione or protein if glucose was also present; glucose alone had a slight effect, insulin alone had none.

These experiments were repeated by Krahl and Penhos (75) using liver slices from both normal and partially depancreatized rats at various times after pancreas removal. The results fully confirmed and extended the earlier work in showing, first, that insulin stimulated amino acid incorporation into liver protein when added in vitro; second, that the effect (for liver slices from fasting animals) depends on the presence of glucose; third, that the stimulation is greater with slices from mildly insulin deficient animals than with those from either normal animals or
long term diabetic animals; and fourth, that this in vitro effect of insulin requires about two or three hours to become maximal.

It is apparent that the action of insulin on protein metabolism is profoundly different in liver and muscle. Whereas the stimulation of incorporation evidenced is dependent on the presence of glucose in the liver, it is independent of glucose concentration in muscle. This suggests a different mode of action in the two tissues.

DISCUSSION

The various metabolic defects found in diabetes mellitus have been discussed at some length. That the secondary effects are important is seen from the statistic that more diabetics die from coronary heart disease due to atherosclerosis than from the actual diabetes itself. This is turn is related to the accompanying disturbances in lipid metabolism rather than to the so-called "primary" disturbance in carbohydrate metabolism. If diabetes mellitus is to be successfully controlled, all of the accompanying metabolic disorders must be taken into account. It is obviously not sufficient that a substance merely lower the blood sugar level for it to be useful in the treatment of the disease; it must also treat the accompanying metabolic derangements of lipid and protein metabolism.
Since diabetes is due to a lack or at least a reduction of native insulin, it is apparent that an increase in insulin should relieve all the symptoms of diabetes. Such has been found to be the case. When insulin is administered to the diabetic, the blood sugar is lowered, the cholesterol in the blood is reduced, ketonemia and ketonuria are abolished; in short, all the diabetic manifestations disappear.

Recently, a number of oral hypoglycemic compounds have been introduced for the treatment of diabetes mellitus. These have done an admirable job in the reduction of the blood sugar level. They have not been investigated very thoroughly in respect to their effect on the other metabolic disorders concerned with diabetes.

Greenberg and associates (53) have reported an increase in the level of plasma beta lipoproteins of diabetics when switched from insulin therapy to an oral hypoglycemic agent (tolbutamide). They interpret this as possibly indicating that the oral hypoglycemic agent does not control the entire diabetic state as adequately as does insulin.

On the other hand, it has been conclusively demonstrated by McDonald and Dalidowicz (95) that the presence of tolbutamide (and several other oral hypoglycemic agents) effectively reduce the in vitro synthesis of cholesterol from acetate and mevalonate
by rat liver homogenates. This would indicate that the oral hypoglycemic agents should be effective in reducing the hypercholesterolemia which accompanies diabetes.

This investigation into the relationship between the oral hypoglycemic agents and protein synthesis is meant to further clarify the role of those compounds in the treatment of diabetes mellitus.
CHAPTER II

THE CHEMISTRY OF INSULIN AND THE ORAL HYPOGLYCEMIC AGENTS

INSULIN

Insulin has been shown to be a protein consisting of two polypeptide chains. The arrangement of amino acids as finally established by Sanger and his co-workers is given in Figure I. The A chain contains 21 amino acids, with glycine at the amino, and asparagine at the carboxyl, end. The B chain has 30 amino acids with phenylalanine at the amino end and alanine at the other. The two chains are joined together by -S-S- linkages at A7-B7 and A20-B19. There is also a disulfide bridge from A6 to All, forming a ring. The amino acids at A8,9,10 can be varied in insulin of various species without demonstrable differences in physiological activity (115,116,117).

DELETION OF AMINO ACIDS

Harris and Li (55) subjected insulin to digestion for six hours with carboxypeptidase. They found that 1.9 moles of alanine and 0.4 moles of asparagine, per 12,000 gm. insulin, had been removed with retention of about 80 per cent of the biological
FIGURE I

THE STRUCTURE OF INSULIN

Glycine

Isoleucine

Valine

Glutamic acid

Glutamine

S

Cysteine

Cysteine—S—S—Cysteine

Alanine

Serine

Valine

S

Cysteine

Serine

Leucine

Tyrosine

Glutamine

Leucine

Glutamic acid

Asparagine

Tyrosine

Cysteine—S—S—Cysteine

Asparagine

A CHAIN

Glycine

Phenylalanine

Valine

Asparagine

Glutamine

Histidine

Leucine

Glycine

Serine

Histidine

Leucine

Valine

Glutamic acid

Alanine

Leucine

Tyrosine

Leucine

Glycine

Glutamic acid

Arginine

Glycine

Phenylalanine

Phenylalanine

Tyrosine

Threonine

Proline

Lysine

Alanine

B CHAIN
activity. Further, with mild trypsin treatment a large fraction of the terminal alanine could be hydrolysed off under conditions where Van Abeele and Campbell (147) reported no loss of biological potency. Harris and Li concluded that the C-terminal alanine in the B chain was not necessary for full biological activity, but that the C-terminal asparagine of the A chain probably was.

Smith et al. (124) treated insulin with leucine aminopeptidase which can attack the insulin structure only at the amino ends of the chains. From this work, it was concluded that the amino acids Bl-B6 were not necessary for full hormonal activity. However, the removal of Al-A5 probably leads to some loss of activity.

From the preceding discussion, it is apparent that the entire insulin molecule is unnecessary for hormonal activity. Exactly how much can be removed while still maintaining physiological action, has not been determined. Such knowledge may yield valuable insight into the mode of action of the hormone.

The actions of the hormone have been treated in the first chapter and will not be reiterated here. Some other experimental findings pertinent to the structure of insulin will be briefly mentioned.

Insulin has been labeled by esterification of the aliphatic hydroxyl groups with $S^{35}O_4$, (48) by iodination of one or more tyrosine residues with $I^{131}$, (43) and by tritiation (149).
The products have not been separated into pure components. This, together with the small number of animals used in assaying the residual biological activity makes it impossible to decide whether the labeled molecules retain biological activity.

The three dimensional structure of insulin in the solid state has been accounted for (82) by assuming that the A and B chains are helices held together by the two disulfide bonds. Each two helices of the 6000 molecular weight structure are then considered to be bonded with two others into a 12,000 molecular weight unit via a zinc ion bridging the B10 and B10' histidine residues of the two sub-units.

The three dimensional structure of insulin in solution depends on a number of factors: the concentration of insulin, the pH, the ionic strength, and the dielectric constant of the medium. In the physiological pH range, molecular (particle) weight values of 24,000 to 48,000 have been estimated (36, 104).

THE ORAL HYPOGLYCEMIC AGENTS

HISTORY

Since insulin is a protein hormone, it is attacked by proteolytic enzymes present in the digestive system with a subsequent loss of activity. It is for this reason that insulin must be injected if it is to be beneficial. Since the disadvantages
of injection were readily apparent, a search for hypoglycemic com-
ounds which could be administered orally has long been in effect. 
As early as 1926, Frank, Nothman, and Wagner (45) reported a hypo-
glycemic effect of a biguanide, synthalin. Further study of this 
compound by Graham and Linder (52) revealed that it caused severe 
liver damage, and it was soon abandoned as a possible insulin sub-
stitute.

For a long time, the extracts of a number of plants have 
been used in many parts of the world as a treatment for diabetes 
mellitus with varying degrees of success. In connection with 
this, Allen (3) investigated the extract of the blueberry leaf and 
Hugh-Jones (61) studied the extracts of periwinkle, mistletoe, and 
nickerberry. The alkaloid, galepine, was described by Muller (102) 
to have the property of reducing the blood sugar of both normal 
and diabetic men. Clementi (31) ascribes similar properties to 
another alkaloid obtained from plants, laparine. A wide range of 
natural sources have yielded compounds with hypoglycemic proper-
ties. Collip (32) extracted a substance, glucokinen, from clams. 
This compound produced a marked hypoglycemic effect when injected 
into rabbits. Similarly, Little, Levine and Best (83) discovered 
an insulin mimetic agent in the disintegration products of killed 
bacteria. Most of these extracts caused liver damage, which fact 
may account for their hypoglycemic effect. At any rate, they are 
useless as therapeutic agents.
Hassal, Reyle, and Fing (56) isolated a hypoglycemic compound, which they called Hypoglycin A, from the fruit of a plant, Blighia sapida. Anderson et al. (4) and Ellington, Hassal, and Plimmer (38) determined that the hypoglycemic activity was due to an unusual amino acid whose structure is given in Table III. A second hypoglycemic compound has been isolated from the same source and designated Hypoglycin B. It is a dipeptide composed of Hypoglycin A and glutamic acid (56).

In 1942, the research on oral hypoglycemic compounds intensified when Janbon, Chaptal, Vadel, and Schaap (66) discovered that a synthetic compound, 5-isopropyl-2-sulfanilamide-1,3,4-thiadiazole (IPTD) could lower blood sugar levels. Although this particular compound proved to be toxic to the point of fatality, its discovery served to open an era of research on synthetic oral hypoglycemic agents. Since then a wide variety of compounds have been examined for hypoglycemic action.

In the next four years, Loubatieres (86) studied the mode of action of IPTD and other thiadiazoles in dogs and rabbits. He discovered that IPTD fails to produce hypoglycemia if the entire pancreas is removed, but may do so if as little as one-sixth of the gland remains. It appears, therefore, that this substance is not a substitute for insulin but rather enhances the effect of native insulin already present, or else spurs the beta cells to a greater production of insulin. Loubatieres found that IPTD is
### TABLE II
THE ARYLSULFONYLUREAS

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbutamide</td>
<td>( H_2N-C-C-\text{SO}_2\text{NHCONH(CH}_2\text{)}_3\text{CH}_3 )</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>( H_3C-C-C-\text{SO}_2\text{NHCONH(CH}_2\text{)}_3\text{CH}_3 )</td>
</tr>
<tr>
<td>Chlorpropamide</td>
<td>( \text{Cl}-C-C-\text{SO}_2\text{NHCONH(CH}_2\text{)}_2\text{CH}_3 )</td>
</tr>
<tr>
<td>Metahexamide</td>
<td>( H_3C-C-C-\text{SO}_2\text{NHCONH-CHNH}_2 )</td>
</tr>
<tr>
<td>Acetohexamide</td>
<td>( H_3C-CO-C-C-\text{SO}_2\text{NHCONH-C-C-C} )</td>
</tr>
</tbody>
</table>
### TABLE III

GUANIDINE AND SOME DERIVATIVES

\[
\begin{align*}
\text{H}_2\text{N} & - \text{C} - \text{NH}_2 \\
\text{NH} & \\
\text{Synthalin A} & \\
(n = 10) & \\
\end{align*}
\]

\[
\begin{align*}
\text{H}_2\text{N} & - \text{C} - \text{NH} (\text{CH}_2)_n \text{NH} - \text{C} - \text{NH}_2 \\
\text{NH} & \\
\text{Synthalin B} & \\
(n = 12) & \\
\end{align*}
\]

\[
\begin{align*}
\text{C} - \text{C} & \\
\text{C} - \text{C} - \text{NH} (\text{CH}_2)_2 \text{NHCNHCNH}_2 & \\
\text{NH} & \\
\text{Phenethylbiguanide} & \\
\end{align*}
\]

\[
\begin{align*}
\text{H}_3\text{C} & - \text{C} - \text{C} - \text{CH}_2\text{CHCOO}^- \\
\text{NH}_3 & \\
\text{Hypoglycin A} & \\
\end{align*}
\]

\[
\begin{align*}
\text{H}_3\text{C} & - \text{C} - \text{C} - \text{CHNHC}(\text{CH}_2)_2\text{CHCOOH} \\
\text{COOH} & \\
\text{NH}_2 & \\
\text{Hypoglycin B} & \\
\end{align*}
\]

\[
\begin{align*}
\text{H}_2\text{N} & - \text{C} - \text{SO}_2\text{NH} - \text{C} \\
\text{N-N} & \\
\text{IPTD} & \\
\end{align*}
\]
most effective when injected directly into the pancreatic artery and that it acts independently of the nervous system. It also lowers the blood sugar in inverse proportion to the sulfonamide level of the blood, and raises the respiratory quotient after glucose administration. Chen, Anderson, and Maze (29) have reported a hypoglycemic action of 5-cyclopropyl-2-sulfanilamide-1,3,4-thiadiazole in intact rabbits. However, since the thiadiazoles were very toxic they were never put to clinical use.

About this time, the studies of Franke, Achenis, Betram et al. (2,14,44) on a new hypoglycemic compound, 1-buty1-3-sulfonylurea (carbutamide) created still more interest in the field. Again, this compound was found to be toxic and was withdrawn from clinical use, at least in the United States. Since then literally thousands of related compounds have been studied for their hypoglycemic activity (24,94).

Most of the compounds tested were found to be too toxic for human consumption, but three analogues of carbutamide, namely tolbutamide, chlorpropamide, and metahexamide have been subjected to clinical studies and the first two are currently extensively used in the management of mild, adult diabetes mellitus. The production of a new arylsulfonyl urea (acetohexamide) was announced only recently (92).
THE ARYLSULFONYLUREAS

Carbutamide (N-butyl-N'-sulfonylurea) is a white crystalline solid which has the weakly acidic properties normally associated with the sulfonyl group. Thus it readily forms water soluble salts with the alkali metals, and is usually obtained as the sodium salt. The quantitation of free carbutamide may be readily performed by diazotization and coupling reactions (19). The liver partially detoxifies carbutamide by acetylation and the resulting compound is slowly eliminated by the kidneys. As with the other sulfonamides containing a p-amino group, carbutamide has some antibacterial action and impairs the thyroid function (5,21).

The simple replacement of the p-amino group of carbutamide by a methyl group eliminates all toxic properties and gives rise to the hypoglycemic compound most widely used in diabetes therapy, tolbutamide (N-butyl-N'-p-toluenesulfonylurea, Orinase). The change of the substituted group on the benzene ring is responsible for many important chemical differences. Tolbutamide is a white crystalline solid with a melting point of 128.5° - 129.5°C, and is virtually insoluble in water, although it forms soluble salts with alkalis. It is readily soluble in the usual organic solvents. Various methods for the determination of tolbutamide in blood and urine are reported in the literature.

In man, tolbutamide is metabolized to the carboxylic
acid obtained when the p-methyl group is oxidized (87); The resulting compound (N-butyl-N'-p-carboxy-phenylsulfonylurea) is freely soluble in water and completely non-toxic. N-butyl-N'-p-hydroxymethyl-phenyl sulfonyl urea has also been found in blood in very small amounts (114). This compound is probably an intermediate in the oxidation to the carboxylic acid. Finally, Mohnike and Wittenhagen (100) isolated from the urine of dogs a toxic metabolite of tolbutamide, p-toluenesulfonamide and another compound which was later shown to be p-toluenesulfonylurea (101). Figure II summarizes the known metabolic break down of tolbutamide.

Tolbutamide is eliminated by the kidneys, primarily as the carboxylic acid (42). In contrast with carbutamide, neither tolbutamide nor its acid significantly affect thyroid function as assessed by $^{131}$I studies (96). The abolition of the goiter-producing properties of the arylsulfonylureas follows the removal of the p-amino group (91).

Chlorpropamide (N-propyl-N'-p-chlorobenzenesulfonylurea) is a white crystalline compound melting at 127.5 - 128.5°C. It differs from tolbutamide in the replacement of the p-methyl group by a chlorine atom, and of the n-butyl radical by n-propyl.

A single one gram dose of chlorpropamide produced a significantly greater hypoglycemic effect in normal subjects than was produced by an identical amount of tolbutamide (136). The biological half life of chlorpropamide is about 35 hours, which is
FIGURE II

THE METABOLISM OF TOLBUTAMIDE

\[
\begin{align*}
\text{p-tolylsulfonylurea} & \\
\text{tolbutamide} & \\
\text{N-butyl-N'\-p-hydroxymethyl-phenylsulfonylurea} & \\
\text{N-butyl-N'\-p-carboxy-phenylsulfonylurea} & \\
p\text{-toluenesulfonamide}
\end{align*}
\]
approximately ten times that of tolbutamide (129). Evidence indicates that this compound, in contrast to tolbutamide, is probably not metabolized to any appreciable extent before excretion through the kidneys. No definite chromatographic or ultraviolet absorption differences have been found between the pure drug and that in serum or urine (68,128). There is, however, a considerable difference between various species of animals. Using the urine of dogs treated with chlorpropamide-S\textsuperscript{35}, three different chromatographic spots were detected. They were identified as the unchanged compound, p-chlorobenzenesulfonylurea, and p-chlorobenzenesulfonamide. The three compounds accounted for 30, 40, and 20 per cent respectively (68). This serves to illustrate the danger involved in the indiscriminate application of animal experiments to man. It is always necessary to take into account the possibility of interspecies differences. In rabbits, as in man, 80 to 90 per cent of the chlorpropamide administered was found to be excreted unchanged (112).

The increased hypoglycemic activity of chlorpropamide as compared with tolbutamide is probably due to the higher initial and more prolonged blood levels attained than to any inherent increased potency (33,70). Thyroid function as assessed by plasma protein-bound iodine levels and the thyroid uptake of radioactive iodine is not impaired by chlorpropamide.

Metahexamide (N-cyclohexyl-N'-\(3\)-amino-4-toluenesulfo-
nylurea) is a very effective hypoglycemic compound with a duration of action which is longer than that of chlorpropamide. It is about half as effective as chlorpropamide and about five times as effective as tolbutamide in producing sustained blood levels (70). Dogs and rabbits excrete 30 to 35 per cent of the administered dose of metahexamide unchanged in the urine and 40 to 50 per cent as 3-amino-4-benzenesulfonamide (112). Since it was found that metahexamide causes obstructive jaundice (89), the compound has been withdrawn from clinical studies.

The newest of the arylsulfonylureas is acetohexamide (1-(p-acetylbenzenesulfonyl)-3-cyclohexylurea. Its chemical structure differs from that of the other members of this group in the substitution of an acetyl group in the para position of the phenyl ring, and the incorporation of a cyclohexyl group on the urea portion of the molecule. (92). Acetohexamide is a white crystalline powder which is insoluble in water but soluble in alcohol or chloroform. Unlike tolbutamide, the sodium salt of acetohexamide is only sparingly soluble in water. In time activity, acetohexamide falls between tolbutamide and chlorpropamide (92). In the human body it is metabolized by reduction of the p-acetyl group. Both acetohexamide and its reduction products have been shown to possess hypoglycemic activity in man and animals. This activity of its metabolic product prolongs the hypoglycemic activity of acetohexamide and is at least partially responsible
for its relatively long duration of action (135).

It is generally agreed that in mammals the arylsulfonylureas produce hypoglycemia only when some functioning beta cells are present. Houssay and Penhos (60) showed that the presence of some pancreatic tissue is essential for the hypoglycemic effect of the arylsulfonylureas in the same way that Loubatieres (86) demonstrated that this was true for the sulfonylamidothiadiazoles. The arylsulfonylureas reduce the fasting blood sugar level of intact and partially pancreatectomized or mildly alloxandiabetic animals, but they do not do so after total pancreatectomy in dogs, rabbits, rats, or man (99,138), nor in animals made severely diabetic with alloxan.

EXTRAHEPATIC EFFECTS OF ARYLSULFONYLUREAS

The mechanism by which tolbutamide and other hypoglycemic arylsulfonylureas exert their effect is still disputed. All of these compounds are, at best, poorly effective in the absence of functional pancreatic cells. Although the evidence indicates that their administration results in decreased pancreatic insulin (107) and increased insulin-like activity in the pancreatic vein (106,109), many metabolic effects which accompany arylsulfonylurea induced hypoglycemia are so different from those which accompany insulin induced hypoglycemia that some investigators postulate the
presence of major extrahepatic factors of arylsulfonylurea action.

Tolbutamide and chlorpropamide added *in vitro*, using rat liver slices, have been found to decrease epinephrine-induced glycogenolysis (148) and ketogenesis (17). When rat liver homogenates were used, tolbutamide was found to alter a number of enzymatic activities, including at least one transaminase (16), several NAD and NADP requiring systems (128), and the phosphorylase reactivating system (12). Tolbutamide and chlorpropamide administered *in vivo* decreased ketogenesis (18,20). Tolbutamide administered *in vivo*, with the effect measured either *in vivo* or *in vitro*, has been found to: (a) decrease glucose output (119), (b) increase the synthesis of glycogen, fatty acids, and protein, without the corresponding extrahepatic effects seen with insulin (111), (c) decrease the conversion of fructose and galactose to glucose (6, 110), and (d) alter the levels of numerous enzymatic activities, including increased activity of malic enzyme (132).

Chlorpropamide may slightly increase the glycogen content of rat diaphragm, while under similar conditions tolbutamide has no effect (31). Chlorpropamide also moderately depresses glutamic-oxalacetic transaminase activity in the livers of well fed animals (118). These scattered reports are compatible with the hypothesis that the arylsulfonylureas affect enzyme reactions which depend upon pyridine nucleotides as co-factors.
THE GUANIDINE DERIVATIVES

In 1926, Frank, Nothman, and Wagner (45) synthesized Synthalin A after earlier reports stated that the administration of guanidine lowers the blood sugar concentration (134). After very limited clinical trials, this compound was demonstrated to produce histological changes in the liver and kidney, and all further use was discontinued (13).

In 1957, the hypoglycemic effect of phenethylbiguanide (N'-betaphenethylformamidinyliminourea hydrochloride) was described by Shapiro, Parrino and Freedman (121). After investigating nearly two hundred different derivatives of formamidinyliminoureas, they found that phenethylbiguanide was a highly active oral hypoglycemic agent in both normal and alloxan-diabetic animals.

The mode of action of phenethylbiguanide differs markedly from that of the arylsulfonylureas as evidenced by the facts that this compound is an effective hypoglycemic agent in pancreactomized animals (103), in severe alloxan-diabetes (146), and in patients with juvenile or insulin deficient types of diabetes (108). Evidently, phenethylbiguanide does not require that functional pancreatic tissue be present; this excludes the possibility that it acts by stimulating the beta cells to produce more insulin as has been proposed for tolbutamide. Phenethylbiguanide in-
creases the glucose uptake by the rat diaphragm in vitro, while the muscle glycogen content decreases (141). A marked increase in lactic acid production and a decrease in oxygen consumption suggest that phenethylbiguanide stimulates anaerobic glycolysis by inhibiting oxidative enzyme systems.

Wick, Larson and Serif (139), using adipose tissue, have shown that phenethylbiguanide inhibits the oxidation of glucose, acetate and succinate and considerably reduces fat synthesis. In man, the blood pyruvate and lactate levels are raised (39) and there is no change in hepatic vein glucose, urea, pyruvate or lactate, nor in the oxygen consumption of the liver (130). Lactate levels in the blood from the femoral artery also remain unchanged. Hypoglycemia cannot be produced in normal humans by the administration of phenethylbiguanide (39), although with intact animals a decrease in the blood sugar concentration is easily produced. These results suggest that the effects of phenethylbiguanide are largely mediated in the peripheral tissues.

The tissue anoxia suggested by this evidence may be attributed to inhibition of cytochrome oxidase and succinic dehydrogenase (126, 131), but a decrease in oxidative phosphorylation would have the same result. Later work suggests that biguanides inhibit the transfer of energy rich phosphate bonds to adenosine diphosphate (76).
Phenethylbiguanide has also been found to significantly increase the levels of serum inorganic phosphate (122) whereas the arylsulfonylureas decrease the serum inorganic phosphate (35, 49, 64, 129). Williams et al. (141) found that phenethylbiguanide inhibited phosphorylase phosphatase, the enzyme catalyzing the conversion of phosphorylase a to phosphorylase b. They also found that the compound has no effect on the activity of phosphorylase a or phosphorylase b or on the conversion of phosphorylase b to phosphorylase a. An inhibition of phosphorylase phosphatase in vitro would favor an increase in the amount of phosphorylase a present in the tissue. Since phosphorylase a is believed to be the form of the enzyme active in glycogenolysis, this could account for part of the decrease in glycogen produced by phenethylbiguanide.
CHAPTER III
MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURE

Female Sprague-Dawley rats, fed ad libitum, on a standard stock diet, were sacrificed by decapitation. The livers were removed without delay, washed in ice cold buffer, blotted, weighed, and homogenized in cold phosphate buffer. The homogenate was centrifuged at 700g to remove cells, cellular debris, and nuclei. A quantity of the homogenate was then incubated at 37°C for two hours together with a number of cofactors, an oral hypoglycemic agent, and leucine-1-Cl4.

In the earlier experiments it was desired to study the distribution of the label among various protein fractions. Accordingly, the reaction was stopped by the addition of 5% casein hydrolysate, which so diluted the isotope that further incorporation was negligible. In some experiments, the diluted incubation mixture was subjected to sonication to release additional protein into the medium which might be held by the microsomes. After sonication, the mixture was centrifuged at 30,000g in a Spinco Ultracentrifuge (Beckman Instruments Inc., Spinco Division,
Belmont, California) to sediment the particulate protein (consisting almost exclusively of mitochondria and microsomes). An aliquot of the supernatant was precipitated for total protein by the addition of 5% trichloroacetic acid; the remainder of the supernatant was utilized in an attempt to isolate beta-lipoprotein fraction. The fraction which was sedimented in the last centrifugation was resuspended in buffer and an aliquot of this was precipitated by the addition of TCA. All precipitates were then assayed for radioactivity using a thin window Geiger-Mueller counter.

Later experiments were concerned with the effects of the concentration of the oral hypoglycemic agent on the protein synthesis, rather than the distribution of the radioactivity. Here, the incubations were carried out in center well flasks containing potassium hydroxide to trap any carbon dioxide liberated in the course of the reaction. The incubations were stopped by the addition of 10% TCA and the total protein removed from the flask, washed consecutively with 5% TCA, ethanol, and acetone. This was then dissolved in 0.1N NaOH, an aliquot analysed for protein content by the biuret reaction, and an aliquot taken to dryness under a heat lamp to be assayed for radioactivity.

The KOH was quantitatively flushed from the center well of the flask into a test tube containing 10% barium chloride. The resultant precipitate was filtered, washed, and assayed for
radioactivity.

**CHEMICALS**

Adenosine-5'-monophosphate, sodium salt, Sigma grade. Sigma Chemical Co., St. Louis Mo. lot # 113B-7160

Adenosine-5'-triphosphate, disodium salt, Sigma grade. Sigma Chemical Co., St. Louis, Mo. lot # 14B-7090

Barium Chloride, General Chemical Co., New York, N.Y. lot # 14

Casein hydrolysate, acid. Nutritional Biochemicals Corp., Cleveland Ohio lot # 8809

Chlorpropamide, sodium salt. Charles Pfizer and Co. Inc., New York, N.Y. lot # 03-422-02EPD

Citric acid, A.C.S. grade. Allied Chemical and Dye Corp. New York, N.Y. lot # 6156

Cupric sulfate pentahydrate, A.R. Mallinkrodt Chemical Works New York, N.Y. lot # 4844

Ethylenedinitrilo tetraacetic acid disodium salt. The Matheson Co., Inc., Norwood, Ohio lot # 302512

Glucose, C.P. Mallinkrodt Chemical Works, New York, N.Y. lot # 4908

Magnesium chloride, Baker Analyzed Reagent. J.T. Baker Chemical Co., Phillipsburg, N.J. lot # 7117

Nicotinamide, Sigma Chemical Co., St. Louis, Mo. lot # 1238-1810
Nicotinamide adenine dinucleotide, beta, prepared from yeast,
Grade III. Sigma Chemical Co., St. Louis, Mo. lot # 54B-7090

Phenethylbiguanide, hydrochloride. U.S. Vitamin Corp., New York,
N.Y. lot # 21

Phosphotungstic acid, Reagent grade, Merck & Co. Inc.,
Rahway, N.J. lot # 41118

Potassium bicarbonate -A.R. Mallinkrodt Chemical Works,
New York, N.Y. lot # 6748

Potassium Chloride -A.R.- Mallinkrodt Chemical Works, New York,
N.Y. lot # 6858

Potassium hydroxide-U.S.P.- Allied Chemical and Dye Corp.,
New York, N.Y. lot # Go24J.

Potassium phosphate dibasic -A.R.- Mallinkrodt Chemical Works,
New York, N.Y. lot # 7092

Potassium phosphate monobasic -A.R.- Mallinkrodt Chemical Works,
New York, N.Y. lot # 7100

Potassium Sodium Tartrate tetrahydrate -A.R. Mallinkrodt Chemical
Works, New York, N.Y. lot # 2367

Sodium Chloride -A.R. Mallinkrodt Chemical Works,
New York, N.Y. lot # 7581

Sodium hydroxide -A.R. Mallinkrodt Chemical Works,
New York, N.Y. lot # 7708
Trichloroacetic acid -A.R.- Mallinkrodt Chemical Works, New York, N.Y. lot # 2928
Tolbutamide - sodium salt, The Upjohn Company, Kalamazoo, Mich. lot # CJO 11,584-2
Leucine - 1- C\textsuperscript{14} and Leucine - C\textsuperscript{14} (U.L.)

Two isotopes were used in the experiments under consideration. Both were obtained from the New England Nuclear Corp., Boston, Mass. and were supplied as the hydrochloride dissolved in 0.01N. HCl. Since this excess acid might adversely affect the buffer systems in the incubations, they were evaporated to dryness in the original vials under a stream of nitrogen gas. The dry amino acid (present as the hydrochloride) was then dissolved in sufficient doubly-distilled water to give a final activity of 1 \textmu C/50 \textmu l of solution. The aqueous solutions were stored in the freezer at -5°C and thawed immediately before use.

The L - leucine- 1- C\textsuperscript{14} is especially valuable for studies in CO\textsubscript{2} evolution, since it is labeled solely in the carboxyl carbon. Its lot number is 173-72-18a and it has a specific activity of 25.2 millicuries/millimole. The radio chemical purity of the material was ascertained by the manufacturer to be greater than 99% as determined by its radiochromatogram following descending chromatography in a solvent system consisting of 1-butanol: water: acetic acid (5:4:1 v/v/v).
The L-leucine- $^{14}$C (U.L.) was especially selected for incorporation studies into protein since it is available in a much higher specific activity than that labeled solely in the 1 position. Its lot number is 162-246-53 and its specific activity is quoted at 223 millicuries/millimole. Again the radiochromatogram as determined by the manufacturer, indicates a purity in excess of 99%.

**COMPOSITION OF SOLUTIONS**

**Biuret reagent** - 1.50g. of cupric sulfate pentahydrate and 6.0 g. of potassium sodium tartrate were dissolved in about 500 ml. of distilled water, 300 ml. of 10% NaOH was added, and the resulting solution was diluted to 1000 ml. The solution was stored in an opaque polyethylene bottle.

**Buffer solution** - 11.574 g. of $\text{K}_2\text{HPO}_4$ and 1.815 g. of $\text{KH}_2\text{PO}_4$ were dissolved in doubly, glass distilled water and the resultant solution was made up to one liter. The final molarity of the solution was .08 M with respect to phosphate. The pH, as measured by a Beckman pH meter, was 7.4.

**Premix solution** - The composition of the premix, essentially that used by Marsh (93) for his work on incorporation of leucine into lipoprotein, is given in Table IV. Generally in the execution of an experiment, equal quantities of homogenate and
<table>
<thead>
<tr>
<th>SUBSTANCE</th>
<th>MOLARITY</th>
<th>CONC. g/50 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>0.075</td>
<td>0.280</td>
</tr>
<tr>
<td>MgCl$_2$·6H$_2$O</td>
<td>0.01</td>
<td>0.102</td>
</tr>
<tr>
<td>KHCO$_3$</td>
<td>0.015</td>
<td>0.076</td>
</tr>
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<td>Glucose</td>
<td>0.015</td>
<td>0.135</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>0.02</td>
<td>0.122</td>
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</tr>
<tr>
<td>ATP</td>
<td>0.0020</td>
<td>0.062</td>
</tr>
</tbody>
</table>
premix were incubated. In the few experiments where the basic premix differed from this table, it will be noted in the text.

Sodium phosphotungstate -(4%, pH 7.50- 7.60). 4.0 g. of phosphotungstic acid was dissolved in about 15 ml. of 1N NaOH and this was diluted to 100 ml. with distilled water, the pH being adjusted by addition of more base until a final pH or 7.50 to 7.60 was reached.

**PREPARATION OF LIVER HOMOGENATES**

Livers were obtained from rats weighing 200-250 grams which were sacrificed by decapitation. The livers were removed as soon after death as possible, usually within 30-60 seconds. They were then rinsed in ice cold buffer solution, blotted, weighed and ground gently in a Potter-Elvehjem homogenizer at 700 r.p.m. for 10 complete vertical strokes with 3.0 volumes of phosphate buffer. (0.1M, pH 7.4). The liver mash was kept at 0°C during the entire homogenization process. Unbroken cells, tissue debris and nuclei were removed by centrifugation at 700g. for 15 min. at 0°C. in a refrigerated centrifuge. (International Portable Refrigerated Centrifuge Model PR-2 Serial no. 24608M). The homogenate was pipetted into the incubation flasks immediately after centrifugation.
ISOLATION OF BETA LIPOPROTEINS
I. Heparin Precipitation

In the earlier work, an adaptation of the heparin precipitation of Burstein (23) was used. After sonication of the incubation mixture, a quantity of rat serum (4-5 ml.) was added to supply carrier lipoprotein. This was then centrifuged for one hour at 100,000g in the Spinco Ultracentrifuge to remove mitochondria and microsomes and the clear supernatant decanted. To this was added a weight of sucrose equal to the volume of the supernatant. (eg. 15 g. of sucrose would be added to 15 ml of supernatant so the final solution would be 100 g. % in sucrose).

To the sucrose solution was added 1.1 ml. of 2M MgCl2 and 1.1 ml. of a 1% solution of heparin in water. This was allowed to stand at room temperature for 20 min. and then centrifuged for 1 hour at 4000 g. The lipoprotein - heparin complex was found floating on the top of the sucrose after the centrifugation.

The lipoprotein was isolated by filtering the solution under suction in a stainless-steel precipitation apparatus (Tracerlab model E-8B, Tracerlab Inc., Boston, Mass.) fitted with a disc of Whatman # 50 filter paper. The precipitate was then washed with 0.1M MgCl2 to remove excess sucrose and soluble protein. (The magnesium chloride wash was used because the lipoprotein-heparin complex appeared to be stable in that solution
whereas it dissociated appreciably in pure water). At this point, the precipitate was washed thoroughly with trichloroacetic acid solution which served to remove excess MgCl₂ and to denature the protein so that it would be retained by the filter paper through subsequent washing. The denatured protein was then washed with distilled water to remove excess trichloroacetic acid.

Extraction of the lipid from the precipitate was achieved by consecutive washings with ethanol, acetone, and ether. The process could be followed visually by a color change from yellow to white probably due to the removal of carotenoid pigments from the lipoprotein.

The precipitate on the filter disc (consisting solely of the denatured protein moiety of the beta-lipoprotein) was oven dried, weighed, and counted with a thin window type assembly consisting of a Tracermatic SC-83 scaler, Tracerlab SG-100 Multimatic Sample Changer and Tracerlab SC-66 Printing Timer. (Tracerlab Inc., Boston, Mass.)

Although the heparin precipitation was a useable technique, it suffered the severe limitation of the necessity of filtering a concentrated sucrose solution. Because of this difficulty, another isolation technique based on the phosphotungstate precipitation of Burstein (22) was developed and employed in the later experiments.
II. Phosphotungstate Precipitation

The procedure as presented is for a total volume of 14 ml; the volume present after an incubation was stopped by the addition of 5 ml. of 5% casein hydrolysate and 5 ml of carrier serum added. Any change in the volume of solution treated will necessitate a corresponding change in the quantity of reagents added.

To the sonicated, centrifuged, incubation mixture was added 0.75 ml. of 4% sodium phosphotungstate and 0.75 ml. of 2M magnesium chloride. The solution was mixed by inversion and centrifuged immediately at 8000 g for 20 minutes. The lipoprotein appeared as a yellow-orange precipitate in the bottom of the centrifuge tube.

The precipitate was washed with 0.1M MgCl₂ in 1% saline and recentrifuged. It was then dissolved in 5 ml. of 0.2% sodium citrate and reprecipitated by the addition of 0.05 g NaCl and 0.15 ml. of 2M MgCl₂. This process of dissolution and reprecipitation was carried out three times to purify the beta-lipoprotein.

The final precipitate was washed twice with 5% TCA to denature the protein followed by three washes with ethanol, three with acetone, one with benzene, and two with ether to extract the lipid from the lipoprotein. The white denatured protein was then filtered as before onto weighed Whatman # 50 filter paper and counted.
To assess the efficacy of the washes in removing radioactivity not actually incorporated into protein, a 1.0 ml. aliquot of each wash was taken to dryness and counted on the system previously described. The data obtained from this procedure is given in the appendix.

This procedure has the decided advantage over the heparin precipitation in that at each step one is dealing with a precipitate rather than a flocculate. (The heparin-lipoprotein complex floats in the sucrose solution). It is then possible to merely centrifuge and decant each wash.

**CARDIAC PUNCTURE**

The serum which was to serve as a source of carrier lipoprotein was obtained by performing cardiac puncture on 200-300 g. rats. The blood thus obtained was allowed to clot, centrifuged to separate the red blood cells from the serum, and stored in the cold until used. In obtaining the blood samples the rat was anaesthetized with ether until it ceased struggling. A 3/4 inch, 25 gauge needle attached to a five c.c. syringe was introduced immediately distal to the sternum and pushed forward through the chest cavity. The needle was introduced completely into the rat and then with the syringe plunger slightly extended, was slowly withdrawn from the rat. When the point of the needle
was within the heart, the syringe began to fill with blood. The syringe was maintained in that position until a 1-2 ml. sample of blood had been obtained.

PROTEIN ISOLATION

In the earlier work, an aliquot of the homogenate (usually 1.0 ml.) was pipetted into a glass stoppered centrifuge tube, 4.0 ml. of 5% TCA were added and the resultant precipitate centrifuged down. It was then washed consecutively with 5% TCA, 95% ethanol, acetone benzenone and ether. After the final ether wash, the white precipitate was re-suspended in 5.0 ml. of ether and filtered through a weighed disc of Whatman # 50 filter paper using the stainless-steel precipitation apparatus previously mentioned. The amount of protein present was determined simply as dry weight of protein - TCA precipitate. There were two principal disadvantages to this procedure; the determination of protein on a dry weight basis was not sufficiently accurate and the geometry of the final plated sample was very poor. The precipitate had a strong tendency to crack; this would result in a significant variation in results when the sample was counted.

Accordingly, a different procedure was utilized in later experiments when the reaction was stopped by the addition of TCA. The precipitated protein was transferred quantitatively from the
incubation flask to a glass stoppered centrifuge tube. It was centrifuged and the precipitate washed with 5% TCA. The precipitate was then washed three times with 95% ethanol and three times with acetone. The efficiency of the washes in removing non-incorporated leucine-\textsuperscript{14}C was determined by plating out a 1.0 ml. aliquot of each wash, evaporating it to dryness and counting the dried sample with a thin window Geiger-Mueller counter. The data in Table XXIII in the appendix clearly indicates that the above procedure provides adequate purification.

After the final acetone wash, excess acetone was removed by gentle suction with an aspirator and the precipitate dissolved in 2.0 ml. of 0.1N sodium hydroxide. A 1.0 ml. aliquot of this solution was taken to dryness in a planchet and counted to determine the incorporation into protein. No correction was made for self-absorption due to NaOH, since this was constant for each sample. The geometry of samples so plated was excellent; each planchet contained a uniform thin layer of solid material on the bottom. The amount of protein in each centrifuge tube was determined by means of a biuret reaction, run in duplicate, on the remainder of the solution.

**PROTEIN DETERMINATION**

During some of the earlier experiments, protein was
merely assayed as dry weight of TCA precipitate. Later, this was felt to be too crude for more exact work, and a modification of the biuret determination of Gornall, Baradawill and David was employed (51). One ml. or less of the sample to be measured was placed in a colorimeter tube. To this was added 4.0 ml of biuret reagent and sufficient distilled water to make a total volume of 5.0 ml. and the solution was allowed to stand for twenty minutes before being read in a Klett-Summerson photoelectric colorimeter fitted with a # 54 filter. Blank tubes contained 4.0 ml of biuret reagent, 0.2 ml of 0.1N sodium hydroxide and 0.2 ml of water. Samples tubes contained 0.2 ml of sample, (TCA protein precipitate dissolved in NaOH as described previously), 0.8 ml of water and 4.0 ml of biuret reagent. Standardization tubes contained 1.0 ml of a solution containing 1.0 to 10.0 mg of bovine serum albumin and 4.0 ml of biuret reagent. Protein concentration was expressed in terms of mg protein nitrogen.

All colorimeter tubes were calibrated against each other before use and a blank and a standard were run with each determination. All sample determinations were run in duplicate; the determinations for the standard curve were run in triplicate.

The data for the calibration curve is given in Table V and the standard curve appears in Figure III.
<table>
<thead>
<tr>
<th>mg Protein</th>
<th>Klett Units</th>
<th>Average</th>
<th>Standard Deviation</th>
</tr>
</thead>
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<td>15.0</td>
<td>1.0</td>
</tr>
<tr>
<td>0.5</td>
<td>14.0</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>15.0</td>
<td>1.0</td>
</tr>
<tr>
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</tr>
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</tr>
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<td>1.5</td>
<td>44.0</td>
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<td></td>
</tr>
<tr>
<td>1.5</td>
<td>44.5</td>
<td></td>
<td></td>
</tr>
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<td>2.1</td>
</tr>
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<td>2.0</td>
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<td></td>
<td></td>
</tr>
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<td>61.0</td>
<td>61.0</td>
<td>2.1</td>
</tr>
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<td>77.0</td>
<td>77.2</td>
<td>1.3</td>
</tr>
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</tr>
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<td>92.0</td>
<td>91.8</td>
<td>0.8</td>
</tr>
<tr>
<td>3.0</td>
<td>92.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>91.0</td>
<td>91.0</td>
<td>0.8</td>
</tr>
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<td>121.5</td>
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</tr>
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<td></td>
<td></td>
</tr>
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<td>4.0</td>
<td>121.5</td>
<td>121.5</td>
<td>2.0</td>
</tr>
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<td>2.0</td>
</tr>
<tr>
<td>5.0</td>
<td>150.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>154.0</td>
<td>154.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Incubations in which the amount of C\textsuperscript{14}O\textsubscript{2} evolved was to be determined were carried out in stoppered center well Erlenmeyer flasks (25 ml. capacity). The center well contained a piece of filter paper saturated with 0.2 ml. of 10% KOH to trap the CO\textsubscript{2}; the main body of the flask held the incubation mixture. After the incubation was stopped, each flask was allowed to shake for 30 minutes longer to allow all CO\textsubscript{2} present to come into contact with the KOH.

After the precipitated protein was removed from the flask, the filter paper was extracted from the center well with a pair of forceps and placed in a test tube containing 2 ml. of water. After shaking, the water was decanted into another tube containing 5.0 ml. of 10% barium chloride. The filter paper was washed a total of three times in this manner. The center well itself was washed a total of five times with distilled water and these washing were also added to the BaCl\textsubscript{2}.

The precipitate of BaCO\textsubscript{3} which formed was filtered on pre-weighed Whatman # 50 filter paper using a Tracerlab Precipitation Apparatus, Model B8-B. The precipitate was washed three times with doubly-distilled water, 50-50 (v/v) ethanol-acetone and finally with pure acetone and allowed to dry for about two hours before weighing and counting.
The counting was done with a thin window type assembly consisting of a Tracermatic SC-83 scaler, Tracerlab SG-100 Multimatic sample changer and Tracerlab SC-66 Printing Timer. All samples were corrected for self-absorption and dead time. Corrections for self absorption were made according to the following equation (145)

\[ \frac{I}{I_o} = 1 - \frac{\text{uh}}{u} \]

where \( I \) = observed activity, \( I_o \) = actual activity, \( u = 0.29 \) and \( h = \text{mg/cm}^2 \) of the plated material. The self-absorption correction curve appears in Figure IV and the pertinent data is given in Table VI.

An approximate correction was made for resolving time by adding 0.5% per 1000 cpm to the observed counting rate (28). The day to day performance of the instrument was checked by counting the same uncalibrated \( ^{14} \text{C} \) source with each group of samples.
Figure IV
<table>
<thead>
<tr>
<th>mg BaCO₃</th>
<th>I/I₀</th>
<th>mg BaCO₃</th>
<th>I/I₀</th>
</tr>
</thead>
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<td>0.0-0.4</td>
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<td>6.7-6.8</td>
<td>0.72</td>
</tr>
<tr>
<td>0.5-0.6</td>
<td>0.98</td>
<td>6.9-7.2</td>
<td>0.71</td>
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<tr>
<td>0.7</td>
<td>0.95</td>
<td>7.3-7.7</td>
<td>0.70</td>
</tr>
<tr>
<td>0.8-0.9</td>
<td>0.94</td>
<td>7.8-8.0</td>
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<td>1.0-1.5</td>
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<tr>
<td>1.6-1.7</td>
<td>0.92</td>
<td>8.4-8.6</td>
<td>0.67</td>
</tr>
<tr>
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<td>0.91</td>
<td>8.7-8.9</td>
<td>0.66</td>
</tr>
<tr>
<td>2.0-2.1</td>
<td>0.90</td>
<td>9.0-9.3</td>
<td>0.65</td>
</tr>
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<td>0.89</td>
<td>9.4-9.6</td>
<td>0.64</td>
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<td>9.7-10.0</td>
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<td>10.9-11.2</td>
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</tr>
<tr>
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<td>0.83</td>
<td>11.7-12.1</td>
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</tr>
<tr>
<td>3.9-4.1</td>
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</tr>
<tr>
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<td>17.0</td>
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<tr>
<td>6.4-6.6</td>
<td>0.73</td>
<td>20.0</td>
<td>0.43</td>
</tr>
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CHAPTER IV

EXPERIMENTAL RESULTS

Experiment 1: Change in pH During the Course of the Incubation.

Before any actual incubations were performed, it was decided to determine the pH of the different solutions in order to ascertain whether differences which might be obtained would be due to the presence of the hypoglycemic agent, rather than to an initial difference in pH. Accordingly, the pH of the buffer alone, the premix alone and the homogenate alone were determined by means of a Beckman pH meter.

A simulated incubation was then run containing premix (1.0 ml.) homogenate (1.0 ml of 2:1 v/w rat liver homogenate), and hypoglycemic agent (4 x 10^{-3} M). Only the isotope was omitted from this incubation. Flasks were gently agitated at 37°C for 2 hours and the initial and final pH of each sample recorded. The data are presented in Table VII.

It was felt that the final pH represented too low a value, so the incubation was repeated using a 3:1 v/w homogenate. Again the initial and final pH was measured and the results tabulated. Since the pH change in this case was considerably smaller,
### TABLE VII
THE pH OF THE INCUBATION MIXTURES

I. pH of Constituents of Mixtures

<table>
<thead>
<tr>
<th>Constituent</th>
<th>pH start</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH buffer</td>
<td>7.42</td>
</tr>
<tr>
<td>pH premix</td>
<td>7.33</td>
</tr>
<tr>
<td>pH homogenate (2:1)</td>
<td>7.04</td>
</tr>
<tr>
<td>pH homogenate (3:1)</td>
<td>7.23</td>
</tr>
</tbody>
</table>

II. pH of Incubation Mixtures Using 2:1 Homogenates

<table>
<thead>
<tr>
<th>Addition</th>
<th>pH start</th>
<th>pH finish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolbutamide</td>
<td>7.04</td>
<td>6.86</td>
</tr>
<tr>
<td>Chlorpropamide</td>
<td>7.02</td>
<td>6.82</td>
</tr>
<tr>
<td>Phenethyllbiguanide</td>
<td>7.02</td>
<td>6.59</td>
</tr>
</tbody>
</table>

III. pH of Incubation Mixtures Using 3:1 Homogenates

<table>
<thead>
<tr>
<th>Addition</th>
<th>pH start</th>
<th>pH finish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolbutamide</td>
<td>7.23</td>
<td>7.02</td>
</tr>
<tr>
<td>Chlorpropamide</td>
<td>7.21</td>
<td>7.01</td>
</tr>
<tr>
<td>Phenethyllbiguanide</td>
<td>7.23</td>
<td>6.80</td>
</tr>
</tbody>
</table>

IV. pH of Homogenates Prepared at Different Times—Constancy of pH

<table>
<thead>
<tr>
<th>Date</th>
<th>Type</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>8/11/64</td>
<td>rat liver</td>
<td>7.23</td>
</tr>
<tr>
<td>8/13/64</td>
<td>rat liver</td>
<td>7.08</td>
</tr>
<tr>
<td>8/14/64</td>
<td>rat liver</td>
<td>7.18</td>
</tr>
<tr>
<td>8/19/64</td>
<td>dog liver</td>
<td>7.18</td>
</tr>
<tr>
<td>9/14/64</td>
<td>rat liver</td>
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<tr>
<td>10/15/64</td>
<td>rat (2) liver</td>
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</tr>
<tr>
<td>11/4/64</td>
<td>rat liver</td>
<td>7.22</td>
</tr>
</tbody>
</table>
it was decided that the 3:1 homogenate would be used in all succeeding experiments.

The observation (Table VII) that there is a greater pH drop in the flask containing phenethylbiguanide than in the flasks containing the sulfonylureas is compatible with the observations of Dalidowicz (34). This must imply that phenethylbiguanide undergoes some reaction, peculiar to that agent alone, which releases hydrogen ion into the medium. This represents just one more indication that the mode of action of phenethylbiguanide is entirely unlike that of the arylsulfonylureas.

Experiment 2: The Incorporation of Leucine-\textsuperscript{C}\textsubscript{14} into Beta-Lipoprotein: Effect of Differing Isotope.

Incubations were run in duplicate at 37°C. for a period of two hours. One incubation flask was kept at 0°C. to serve as a control. Radioactivity present in the lipoprotein above that present in the 0°C flask was interpreted as representing incorporation. The incubations were run using 2 \textmu c of either leucine-l-\textsuperscript{C}\textsubscript{14} (U.L.), the latter possessing a much higher specific activity. Lipoprotein was separated by the heparin technique of Burstein (23).

The results in Table VIII indicate that a very small amount of incorporation occurred using the isotope of lower specific activity. A more significant amount of incorporation was
**TABLE VIII**

**INCORPORATION OF LEUCINE INTO BETA-LIPOPROTEIN:**

**EFFECT OF SPECIFIC ACTIVITY OF ISOTOPE.**

<table>
<thead>
<tr>
<th>Incub. temp</th>
<th>Leucine-1-$\text{C}^{14}$ (sp. act. 25mc/mm)</th>
<th>Leucine-$\text{C}^{14}$ (U.L.) (sp. act. 233mc/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>36 cpm</td>
<td>112 cpm</td>
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<tr>
<td>37°C</td>
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</tr>
<tr>
<td>background</td>
<td>20 cpm</td>
<td>18 cpm</td>
</tr>
</tbody>
</table>
evident when the leucine with the higher specific activity was used, but the results were not very reproducible.

**Experiment 3: The Incorporation of Leucine - $^{14}C$ into Beta-Lipoprotein. Effect of Tolbutamide Addition and of Omission of Premix**

Each incubation flask contained 2 uc of leucine - $^{14}C$ (U.L.) in a total volume of 2.0 ml. Tolbutamide was added to two flasks as the sodium salt, to give a final concentration of $4 \times 10^{-3}$ M. In two other flasks the 1.0 ml of premix was replaced by an identical amount of distilled water. The incubation was conducted for two hours, at $37^\circ$C and the contents sonicated for 30 min. before the lipoprotein was isolated by heparin precipitation.

The data (Table IX) are not sufficiently accurate to determine the effect of tolbutamide on the beta-lipoprotein synthesis. There may be a slight inhibition, but this is uncertain. However, it is apparent that the omission of the premix results in a lowered incorporation of isotope into the lipoprotein. This observation substantiates the work of Marsh (93) who first demonstrated incorporation into lipoprotein. Since Marsh separated lipoprotein ultracentrifugally, this substantiation of his results by heparin precipitation would indicate that there is genuine synthesis taking place; the radioactivity associated with the lipoprotein is not merely an artifact of the method. However,
## TABLE IX

**INCORPORATION OF LEUCINE INTO BETA-LIPOPROTEIN:**

**EFFECT OF TOLBUTAMIDE ADDITION AND OF OMISSION OF PREMIX**

<table>
<thead>
<tr>
<th>Experimental Procedure</th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C incubation</td>
<td>80</td>
</tr>
<tr>
<td>37°C incubation</td>
<td>50</td>
</tr>
<tr>
<td>Tolbutamide added</td>
<td>58</td>
</tr>
<tr>
<td>Tolbutamide added</td>
<td>35</td>
</tr>
<tr>
<td>Premix omitted</td>
<td>30</td>
</tr>
<tr>
<td>Premix omitted</td>
<td>29</td>
</tr>
<tr>
<td>0°C control</td>
<td>19</td>
</tr>
<tr>
<td>Background</td>
<td>20</td>
</tr>
</tbody>
</table>
although the isotope is definitely incorporated into the lipoprotein, the resultant activity is not sufficient to yield reproducible data.

**Experiment 4: Distribution of Radioactivity in Various Protein Fractions.**

Since there was apparently very little label being incorporated into the beta-lipoprotein fraction, it was decided to investigate the relative amount found in various protein fractions. Four flasks were incubated; 2 controls, 1 containing $4 \times 10^{-3}$ M tolbutamide, and 1 containing $4 \times 10^{-3}$ M orotic acid. This last chemical was employed because it has been postulated to inhibit hepatic lipoprotein secretion (144).

Each incubation flask contained 2.0 ml of premix, 2.0 ml. of homogenate and 10 ucuries of leucine-C$^{14}$ (U.L.). After 2 hrs. incubation at 37°C, each flask was stopped by the addition of 5.0 ml. of 5% casein hydrolysate. After sonication, the contents were centrifuged for 1 hour at 100,000g. to sediment mitochondria and microsomes. These particles represent one fraction which was studied. They were resuspended in 4.0 ml. of buffer by gentle homogenization and a 1.0 ml. aliquot of the suspension was precipitated with TCA, washed, evaporated to dryness and counted.

A 1.0 ml. aliquot of the supernatant liquid (which was
separated from the sedimented particles by decantation) was precipitated for protein by the addition of 4.0 ml. of 5% TCA, washed and counted.

Carrier plasma was added to the remainder of the supernatant and the beta-lipoproteins were isolated by phosphotungstate precipitation, washed and counted. Thus the original incubation mixture was separated into 3 components: The particulate proteins, the total soluble proteins and the beta-lipoproteins. This separation is illustrated schematically in Figure V.

It is apparent from the data in Table X that there is a very significant degree of incorporation of leucine in both the soluble protein and the particulate protein. Furthermore, tolbutamide inhibits the incorporation into both fractions to approximately the same extent. The presence of orotic acid has no effect on incorporation into either fraction. The data concerning lipoprotein incorporation is less certain; the amount of activity present is still not large. No attempt was made to calculate the specific activity of the lipoprotein, since the amount isolated was too small to measure accurately. It appears that both tolbutamide and orotic acid inhibit lipoprotein synthesis, but the proof is far from conclusive.
FIGURE V

SEPARATION OF PROTEIN FRACTIONS

INCUBATION MIXTURE

Stop Rx. by addition of 5.0 ml. 5% casein hydrolysate
Add 1.0 ml. serum sonicate
Centrifuge 100,000 g.

Mitochondria
microsomes

5% TCA

particulate
protein
ppt.

Supernatant (10.0 ml)

Divide in
two parts

1.0 ml.
9.0 ml.

4.0 ml.
Add 5.0 ml. Carrier serum

5% TCA

soluble prot.
ppt.

Phosphotung-
state

beta-lipoprotein
ppt.
### TABLE X

**DISTRIBUTION OF RADIOACTIVITY**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Weight mg.</th>
<th>Counts</th>
<th>Time (min)</th>
<th>cpm</th>
<th>cpm/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein-soluble</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no addition</td>
<td>4.8</td>
<td>3000</td>
<td>7.22</td>
<td>416</td>
<td>87</td>
</tr>
<tr>
<td>no addition</td>
<td>4.6</td>
<td>3000</td>
<td>6.62</td>
<td>453</td>
<td>98</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>4.0</td>
<td>3000</td>
<td>11.64</td>
<td>258</td>
<td>65</td>
</tr>
<tr>
<td>orotic acid</td>
<td>4.8</td>
<td>3000</td>
<td>7.14</td>
<td>420</td>
<td>88</td>
</tr>
<tr>
<td><strong>Protein-particulate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no addition</td>
<td>9.0</td>
<td>3000</td>
<td>0.79</td>
<td>3797</td>
<td>422</td>
</tr>
<tr>
<td>no addition</td>
<td>8.7</td>
<td>3000</td>
<td>0.99</td>
<td>3030</td>
<td>348</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>10.1</td>
<td>3000</td>
<td>1.41</td>
<td>2128</td>
<td>211</td>
</tr>
<tr>
<td>orotic acid</td>
<td>9.1</td>
<td>3000</td>
<td>0.96</td>
<td>3125</td>
<td>343</td>
</tr>
<tr>
<td><strong>Beta-lipoprotein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no addition</td>
<td>-</td>
<td>3000</td>
<td>31.12</td>
<td>96</td>
<td>-</td>
</tr>
<tr>
<td>no addition</td>
<td>-</td>
<td>3000</td>
<td>34.13</td>
<td>88</td>
<td>-</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>-</td>
<td>3000</td>
<td>54.81</td>
<td>55</td>
<td>-</td>
</tr>
<tr>
<td>orotic acid</td>
<td>-</td>
<td>3000</td>
<td>85.47</td>
<td>35</td>
<td>-</td>
</tr>
</tbody>
</table>
Experiment 5: A Comparison of the Effect of Tolbutamide and Phenethylbiguanide on the Incorporation of Leucine into Various Protein Fractions.

The incubations and subsequent separations were carried out precisely as in the preceding experiment. Data for this experiment are presented in Table XI. These results confirm the previous observations that tolbutamide inhibits incorporation of the isotope into both the soluble and the particulate protein. In contrast to this, phenethylbiguanide does not appear to have an inhibitory effect in either fraction. It appears that tolbutamide might have an inhibitory effect in the beta-lipoprotein fraction, but phenethylbiguanide probably does not. A concise summary of the data obtained from the last two experiments is presented in Table XII.

Since a very early step in the catabolism of leucine (Figure VI) involves a decarboxylation reaction, it was decided to trap the CO₂ liberated in the course of the incubations and to determine the effect of tolbutamide and phenethylbiguanide on leucine catabolism as well as anabolism. For the first experiments, leucine - l C¹⁴ was employed, so that there would be a strict one-to-one correspondence between the amount of leucine catabolized and the amount of C¹⁴O₂ produced.
TABLE XI

DISTRIBUTION OF RADIOACTIVITY

INCUBATION WITH TOLBUTAMIDE AND PHENETHYLBIGUANIDE

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Weight mg.</th>
<th>Counts</th>
<th>Time (min)</th>
<th>cpm</th>
<th>cpm/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein-soluble</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no addition</td>
<td>11.0</td>
<td>3000</td>
<td>3.18</td>
<td>943</td>
<td>86</td>
</tr>
<tr>
<td>no addition</td>
<td>11.0</td>
<td>3000</td>
<td>2.62</td>
<td>1145</td>
<td>104</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>9.8</td>
<td>3000</td>
<td>5.07</td>
<td>592</td>
<td>60</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>9.3</td>
<td>3000</td>
<td>5.29</td>
<td>567</td>
<td>61</td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>9.6</td>
<td>3000</td>
<td>3.27</td>
<td>917</td>
<td>96</td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>10.3</td>
<td>3000</td>
<td>3.03</td>
<td>990</td>
<td>96</td>
</tr>
<tr>
<td><strong>Protein-particulate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no addition</td>
<td>5.3</td>
<td>10000</td>
<td>1.97</td>
<td>5076</td>
<td>958</td>
</tr>
<tr>
<td>no addition</td>
<td>6.0</td>
<td>10000</td>
<td>1.85</td>
<td>5368</td>
<td>964</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>6.2</td>
<td>10000</td>
<td>2.71</td>
<td>3690</td>
<td>595</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>5.9</td>
<td>10000</td>
<td>2.57</td>
<td>3891</td>
<td>659</td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>5.2</td>
<td>10000</td>
<td>1.85</td>
<td>5405</td>
<td>1039</td>
</tr>
<tr>
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<td>10000</td>
<td>1.57</td>
<td>6369</td>
<td>1098</td>
</tr>
<tr>
<td><strong>Beta-lipoprotein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no addition</td>
<td>8.3</td>
<td>3000</td>
<td>7.62</td>
<td>394</td>
<td>47</td>
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<td>no addition</td>
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<td>3000</td>
<td>8.97</td>
<td>334</td>
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<td>tolbutamide</td>
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<td>18.64</td>
<td>161</td>
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<td>tolbutamide</td>
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<td>3000</td>
<td>8.57</td>
<td>350</td>
<td>35</td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>7.0</td>
<td>3000</td>
<td>8.99</td>
<td>334</td>
<td>48</td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>11.2</td>
<td>3000</td>
<td>5.50</td>
<td>545</td>
<td>49</td>
</tr>
<tr>
<td>Fraction</td>
<td>Experiment 4</td>
<td></td>
<td>Experiment 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------</td>
<td>-------</td>
<td>--------------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average sp. act.</td>
<td>% of Control</td>
<td>Average sp. act.</td>
<td>% of Control</td>
<td></td>
</tr>
<tr>
<td>Protein-soluble</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>93</td>
<td>100</td>
<td>95</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>tolbutamide</td>
<td>65</td>
<td>70</td>
<td>60</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>-</td>
<td>-</td>
<td>96</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Protein-particulate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>385</td>
<td>100</td>
<td>961</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>tolbutamide</td>
<td>211</td>
<td>55</td>
<td>528</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>-</td>
<td>-</td>
<td>1003</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>Beta-lipoprotein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>-</td>
<td>-</td>
<td>45</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>tolbutamide</td>
<td>-</td>
<td>-</td>
<td>38</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>-</td>
<td>-</td>
<td>48</td>
<td>106</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE VI

THE CATABOLISM OF LEUCINE (137)

\[
\begin{align*}
H_3C & \quad \text{NH}_2 \quad \text{CH}CH_2CHCOOH \\
\downarrow & \quad \text{transamination} \\
H_3C & \quad \text{O} \quad \text{CHCH}_2C\text{COOH} \\
\downarrow & \quad \text{CoASH} \\
H_3C & \quad \text{O} \quad \text{CHCH}_2CSCoA + C^*O_2 \\
\downarrow & \quad -2\text{H} \\
H_3C & \quad \text{O} \quad \text{CHCHCSCoA} \\
\downarrow & \quad \text{CO}_2 \quad \text{ATP} \\
\text{HOOC-CH}_2 & \quad \text{O} \quad \text{CCHCSCoA} \\
\downarrow & \quad \text{H}_2\text{O} \\
\text{HOOC-CH}_2 & \quad \text{OH} \quad \text{CCHCSCoA} \\
\downarrow & \quad \text{} \\
\text{HOOC-CH}_2 & \quad \text{O} \quad \text{H}_3C-CCH_2COOH \\
\downarrow & \quad \text{} \\
\text{HOOC-CH}_2 & \quad \text{O} \quad \text{H}_3C-CCHCSCoA \\
\downarrow & \quad \text{H}_3C-C-C\text{SCO}_2A \quad \text{H}_3C-C-C\text{SCO}_2A \\
\end{align*}
\]
Experiment 6: Effect of Tolbutamide and Phenethylbiguanide on 
$^{14}CO_2$ Production from Leucine - 1 - $^{14}C$.

For this experiment, five flasks were incubated as usual. Two contained 4 umoles of tolbutamide, two contained 4 umoles of phenethylbiguanide, one contained no added agent. The CO$_2$ liberated in the course of the incubation was trapped in KOH in the center-well of the incubation flask. This was isolated as BaCO$_3$ and counted. The incubation was stopped after two hours by the addition of 0.5 ml. of 10% TCA and the resulting protein precipitate was likewise isolated and counted. The data is tabulated in Table XIII.

It will be noted that the CO$_2$ data are expressed as total cpm rather than as specific activity (cpm/mg). Since the reason for this is not immediately apparent, a brief discussion might be useful. Since the total CO$_2$ comes from many places other than just the decarboxylation of leucine (e.g. Krebs cycle), the specific activity is a meaningless expression. This is so because the effect of the oral hypoglycemic agents on these other sources of CO$_2$ is not known and the specific activity will necessarily vary as the total weight of CO$_2$ (as BaCO$_3$) varies. Thus the specific activity would be different if tolbutamide were to depress the Krebs cycle from if it were to accelerate it.

To clarify the above, the following hypothetical case is
TABLE XIII

CO₂ RELEASE AND PROTEIN INCORPORATION FROM EXPERIMENT 6

CO₂ DATA (CATABOLISM)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight mg.</th>
<th>Counts</th>
<th>Time (min)</th>
<th>cpm</th>
<th>cpm corr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>7.3</td>
<td>300K</td>
<td>9.61</td>
<td>31,200</td>
<td>51,550</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>5.5</td>
<td>300K</td>
<td>8.41</td>
<td>35,700</td>
<td>55,400</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>5.7</td>
<td>300K</td>
<td>7.80</td>
<td>38,500</td>
<td>60,400</td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>4.1</td>
<td>300K</td>
<td>17.59</td>
<td>17,000</td>
<td>22,500</td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>4.7</td>
<td>300K</td>
<td>17.00</td>
<td>17,600</td>
<td>24,300</td>
</tr>
</tbody>
</table>

PROTEIN INCORPORATION (ANABOLISM)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight mg.</th>
<th>Counts</th>
<th>Time (min)</th>
<th>cpm</th>
<th>cpm/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>4.8</td>
<td>10K</td>
<td>7.56</td>
<td>1320</td>
<td>276</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>4.6</td>
<td>10K</td>
<td>18.38</td>
<td>543</td>
<td>118</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>5.0</td>
<td>10K</td>
<td>14.64</td>
<td>682</td>
<td>136</td>
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<tr>
<td>phenethylbiguanide</td>
<td>5.1</td>
<td>10K</td>
<td>9.97</td>
<td>1000</td>
<td>196</td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>4.8</td>
<td>10K</td>
<td>9.10</td>
<td>1100</td>
<td>229</td>
</tr>
</tbody>
</table>
considered. Substance x depresses the catabolism of leucine only and has no effect on other sources of CO₂. Substance y depresses the catabolism of leucine but also depresses CO₂ production via the Krebs cycle.

<table>
<thead>
<tr>
<th></th>
<th>cpm</th>
<th>CO₂ from leucine</th>
<th>Other CO₂</th>
<th>cpm/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>100</td>
<td>5 mg</td>
<td>5 mg</td>
<td>10</td>
</tr>
<tr>
<td>x added</td>
<td>25</td>
<td>1.25 mg</td>
<td>5 mg</td>
<td>4</td>
</tr>
<tr>
<td>y added</td>
<td>25</td>
<td>1.25 mg</td>
<td>1.25 mg</td>
<td>10</td>
</tr>
</tbody>
</table>

In this example, substance y reduces the decarboxylation of leucine by a factor of 4. This effect is not evident from a consideration of the specific activities because it also reduces the amount of CO₂ produced from other sources.

From the data obtained in experiment 6, it is apparent that tolbutamide has little effect on the catabolism of leucine, whereas phenethylbiguanide has a very significant inhibitory effect. The previously observed effects on protein synthesis are verified in this experiment; phenethylbiguanide has little effect while tolbutamide exerts a significant inhibition.

From the data in this experiment, the ratio of catabolism to anabolism can be calculated.

\[
\frac{\text{catabolism}}{\text{anabolism}} = \frac{51,500}{1320} = 39
\]
This result indicates that for each molecule of leucine incorporated into protein under control conditions, 39 molecules are being broken down. The system under study is a highly catabolic one.

**Experiment 7: The Effect of Three Hypoglycemic Agents on Leucine Anabolism and Catabolism.**

This experiment was designed to be a verification of the previous observations, with the inclusion of chlorpropamide effects on protein incorporation and CO₂ release. The incubation flasks contained 0.5 ml. of homogenate, 0.5 ml. of premix, 1 μc of leucine-1-C¹⁴, and sufficient oral hypoglycemic agent to make the final concentration 4 x 10⁻³ M. The hypoglycemic agents were added in 1.0 ml. of water, making a total volume in each incubation flask of 2.0 ml. The experimental flasks incubated consisted of: 1 kill flask to which was added 0.5 ml. of 10% TCA before incubation, 1 control flask containing no hypoglycemic agent, and 2 each of flasks containing 4 x 10⁻³ M tolbutamide, chlorpropamide and phenethylbiguanide. Data are presented in Table XIV.

The data on the release of C¹⁴O₂ verify that obtained in experiment 6. The presence of tolbutamide exerts little or no effect on the catabolism of leucine. Phenethylbiguanide has a significant inhibitory influence on it and chlorpropamide has an
**TABLE XIV**

**EFFECT OF THREE AGENTS ON ANABOLISM AND CATABOLISM OF LEUCINE**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight mg.</th>
<th>Counts</th>
<th>Time (min)</th>
<th>cpm</th>
<th>cpm corr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>no addition</td>
<td>8.1</td>
<td>100K</td>
<td>8.70</td>
<td>11,494</td>
<td>17,749</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>5.6</td>
<td>100K</td>
<td>8.25</td>
<td>12,121</td>
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<td>12.50</td>
<td>8,000</td>
<td>11,093</td>
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</table>

**CO₂ DATA**

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<th>Time (min)</th>
<th>cpm</th>
<th>cpm/mg</th>
</tr>
</thead>
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</tr>
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<td>10K</td>
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<td>52.97</td>
<td>189</td>
<td>33</td>
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</tbody>
</table>

**PROTEIN DATA**
effect very similar to phenethylbiguanide. This last observation is most surprising, since chlorpropamide is very similar structurally to tolbutamide and totally unlike phenethylbiguanide.

The protein data from this experiment are inconclusive, since the specific activities obtained are too low to be meaningful. This is evidently a result of the dilution of the incubation mixture (it was one-half as concentrated as in previous experiments) and the fact that only 1 uc. of leucine-1-C\textsuperscript{14} was added to each flask.

**Experiment 8: The effect of the Concentration of Phenethylbiguanide on C\textsuperscript{14}O\textsubscript{2} Evolution.**

The Experimental procedure was similar to that employed in experiment 7, but protein incorporation was not determined. Incubation flasks contained 0.5 ml. premix, 0.5 ml. homogenate and 1 uc of leucine - 1 - C\textsuperscript{14}. Added to this was a varying amount of phenethylbiguanide (from 0-30 umoles) dissolved in 1.0 ml. of H\textsubscript{2}O, so that the total volume of each flask was 2.0 ml. The radioactivity present in the CO\textsubscript{2} collected is given in Table XV.

The inhibition of CO production is illustrated graphically in Figure VII.
<table>
<thead>
<tr>
<th>Umoles of PEBG added</th>
<th>Weight BaCO$_3$ (mg.)</th>
<th>Counts</th>
<th>Time (min)</th>
<th>cpm</th>
<th>cpm corr.</th>
</tr>
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<tbody>
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<td>5930</td>
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<td>4650</td>
<td>6960</td>
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</tr>
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<td>5000</td>
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<td>30K</td>
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<td>2935</td>
<td>4810</td>
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<td>11.62</td>
<td>2580</td>
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<td>30K</td>
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<td>10K</td>
<td>3.51</td>
<td>2850</td>
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</table>
Figure VII

PHENETHYLBIGUANIDE

(M x 10^{-6})
Experiments 9 & 10: The Effect of the Concentration of Phenethylbiguanide on $^{14}\text{C}_2$ Evolution and Protein Incorporation.

Incubations were performed using 0.5 ml. of homogenate, 0.5 ml. of premix, 2 ucuries of leucine-1-$^{14}\text{C}$, and varying amounts of phenethylbiguanide. The total volume in each flask was 1.0 ml. Evolved $\text{CO}_2$ was trapped in the center well and total protein was precipitated and counted. The protein was dissolved in NaOH and plated out as such. Its concentration was measured by means of the biuret reaction.

The data for experiment 9 appears in Tables XVI and XVII. Experiment 10 was designed as a verification of this experiment, and the results are presented in the appendices in Tables XXIV and XXV.

Experiment 11: The Effect of the Concentration of Tolbutamide on $^{14}\text{C}_2$ Evolution and Protein Incorporation.

The experiment was performed exactly as the preceding two, except that tolbutamide was substituted for the phenethylbiguanide. Both protein and $\text{CO}_2$ were collected and counted as before. Results are given in Tables XVIII and XIX. A comparison of the relative effects of tolbutamide and phenethylbiguanide on $\text{CO}_2$ production and protein synthesis is presented in Figures VIII
TABLE XVI

EFFECT OF CONCENTRATION OF PHENETHYL-
BIGUANIDE ON $^{14}O_2$ EVOLUTION (Experiment 9)

<table>
<thead>
<tr>
<th>Umoles of PEBG added</th>
<th>Weight BaCO$_3$ (mg.)</th>
<th>Counts</th>
<th>Time (min)</th>
<th>cpm</th>
<th>cpm corr.</th>
</tr>
</thead>
<tbody>
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TABLE XVII

EFFECT OF CONCENTRATION OF PHENETHYLBIGUANIDE ON INTEGRATION OF LEUCINE-1-C$^{14}$ INTO PROTEIN OF RAT LIVER.

<table>
<thead>
<tr>
<th>Umoles of PEBG added</th>
<th>Weight Protein mg.</th>
<th>Counts</th>
<th>Time (min)</th>
<th>cpm</th>
<th>cpm/mg</th>
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<td>10K</td>
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<td>Weight BaCO₃ (mg.)</td>
<td>Counts</td>
<td>Time (min)</td>
<td>cpm</td>
<td>cpm corr.</td>
</tr>
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<td>100K</td>
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<td>100K</td>
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</table>
### TABLE XIX

**EFFECT OF CONCENTRATION OF TOLBUTAMIDE ON INCORPORATION OF LEUCINE-1-C\textsubscript{14} INTO PROTEIN OF RAT LIVER**

<table>
<thead>
<tr>
<th>Umoles of tolbutamide added</th>
<th>Weight Protein mg.</th>
<th>Counts</th>
<th>Time (min)</th>
<th>cpm</th>
<th>cpm/mg</th>
</tr>
</thead>
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<td>4.85</td>
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<td>7.80</td>
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<td>10K</td>
<td>10.82</td>
<td>924</td>
<td>106</td>
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</tbody>
</table>
Figure VIII

- TOLBUTAMIDE
- PEBG

HYPOGLYCEMIC AGENT (µM)
HYPOGLYCEMIC AGENT (μM)

Figure IX
It is clearly evident that the inhibition of CO$_2$ release by tolbutamide is linear with respect to concentration of the oral hypoglycemic agent, while the inhibition by phenethylbiguanide is exponential in character, reaching a relatively constant level at a concentration of about 8 umoles of agent. Further, a considerably greater inhibition by phenethylbiguanide is evident at all levels of the agents utilized. These facts indicate that the inhibition by the two hypoglycemic agents is brought about by different mechanisms.

Likewise, the data on the incorporation of leucine-1-$^{14}$C into protein show a remarkable difference in the action of the two chemicals. The inhibition by tolbutamide is again linear, although the experimental points do not delineate a very exact line. The inhibition by phenethylbiguanide is of considerable interest. Little or no effect is seen with up to 6 umoles of the compound added. This is in agreement with the earlier experiments which showed no inhibition by phenethylbiguanide. (cf. experiments 4, 5 and 6). However, as the concentration of phenethylbiguanide approaches 8 umoles a very significant drop in incorporation occurs. This levels out at a concentration of 10 umoles and no further change is evidenced when 15 umoles of agent are added. Thus a considerable inhibition occurs in the range of 8 umoles of PEBG. This observation was verified in experiment 10.
which is given in the appendix in Tables XXIV and XXV.

One possible explanation for this phenomenon is that there is some substance present in the incubation mixture which ties up the phenethylbiguanide so that it cannot inhibit protein synthesis. According to this view, once 8 umoles of phenethylbiguanide are present, all of the unknown substance is tied up and the excess phenethylbiguanide is free to act as an inhibitor. Thus if the hypothetical inactivating compound were absent entirely, even a small amount of PEBG would inhibit protein incorporation almost completely.

Since the most obvious effect of phenethylbiguanide is a lowering of blood sugar, it was postulated that the PEBG combined with glucose to give a product which would not affect protein synthesis. Once all the glucose in the medium was utilized, the free phenethylbiguanide could create its effect. Since the total amount of glucose added to each flask in the premix was 8 umoles, this seemed to be a reasonable postulate. The amounts used were such that PEBG and glucose could react in a stoichiometric 1:1 relationship to form an inactive PEBG-glucose complex. The next experiment was designed to test this hypothesis.
Experiment 12: The Effect of Glucose on Inhibition by Hypoglycemic Agents of Leucine Anabolism and Catabolism.

The following mixtures were incubated, in duplicate, under the usual conditions:

control- no glucose; no additive
control plus glucose- contained glucose; no additive
tolbutamide- no glucose; tolbutamide added
tolbutamide plus glucose
phenethylbiguanide- no glucose; PEBG added
phenethylbiguanide plus glucose

The premix was prepared as usual, except that no glucose was added; instead, 8 umoles were added separately to those flasks requiring glucose. The concentration of hypoglycemic agent was 4 umoles/ml. Each flask contained 0.5 ml. of premix, 0.5 ml. of homogenate, and 2 uc. of leucine- C$^{14}$ (U.L.). Results appear in Tables XX and XXI.

It is immediately obvious from the data that the presence of glucose affects neither CO$_2$ release nor incorporation into protein. All results obtained with added glucose are essentially the same as those seen in the absence of glucose.
TABLE XX

EFFECT OF GLUCOSE AND ORAL HYPOGLYCEMIC AGENTS ON $^{14}C_{2}$ RELEASE

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Weight BaCO$_3$ mg.</th>
<th>Counts</th>
<th>Time (min)</th>
<th>cpm</th>
<th>cpm corr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>11.5</td>
<td>100K</td>
<td>13.46</td>
<td>7429</td>
<td>12,592</td>
</tr>
<tr>
<td>control</td>
<td>13.1</td>
<td>100K</td>
<td>14.26</td>
<td>7013</td>
<td>12,751</td>
</tr>
<tr>
<td>control - glucose</td>
<td>9.7</td>
<td>100K</td>
<td>13.21</td>
<td>7570</td>
<td>12,159</td>
</tr>
<tr>
<td>control - glucose</td>
<td>9.2</td>
<td>100K</td>
<td>11.89</td>
<td>8410</td>
<td>12,938</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>9.8</td>
<td>100K</td>
<td>15.22</td>
<td>6570</td>
<td>10,429</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>10.0</td>
<td>100K</td>
<td>15.06</td>
<td>6640</td>
<td>10,540</td>
</tr>
<tr>
<td>tolbutamide - glucose</td>
<td>7.7</td>
<td>100K</td>
<td>15.00</td>
<td>6666</td>
<td>9,523</td>
</tr>
<tr>
<td>tolbutamide - glucose</td>
<td>10.7</td>
<td>100K</td>
<td>17.01</td>
<td>5879</td>
<td>9,638</td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>8.6</td>
<td>100K</td>
<td>32.69</td>
<td>3059</td>
<td>4,566</td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>8.3</td>
<td>100K</td>
<td>29.53</td>
<td>3386</td>
<td>4,979</td>
</tr>
<tr>
<td>PEBG - glucose</td>
<td>8.1</td>
<td>100K</td>
<td>31.05</td>
<td>3221</td>
<td>4,737</td>
</tr>
</tbody>
</table>
TABLE XXI

EFFECT OF GLUCOSE AND ORAL HYPOGLYCEMIC AGENTS
ON INCORPORATION OF LEUCINE-$^{14}$ INTO PROTEIN

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Weight Protein mg.</th>
<th>Counts</th>
<th>Time (min)</th>
<th>cpm</th>
<th>cpm/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>6.90</td>
<td>30K</td>
<td>13.31</td>
<td>2254</td>
<td>327</td>
</tr>
<tr>
<td>control</td>
<td>6.40</td>
<td>30K</td>
<td>14.08</td>
<td>2131</td>
<td>333</td>
</tr>
<tr>
<td>control - glucose</td>
<td>7.10</td>
<td>30K</td>
<td>12.02</td>
<td>2496</td>
<td>352</td>
</tr>
<tr>
<td>control - glucose</td>
<td>6.65</td>
<td>30K</td>
<td>11.50</td>
<td>2609</td>
<td>392</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>6.90</td>
<td>30K</td>
<td>18.86</td>
<td>1591</td>
<td>231</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>7.10</td>
<td>30K</td>
<td>18.55</td>
<td>1617</td>
<td>228</td>
</tr>
<tr>
<td>tolbutamide - glucose</td>
<td>6.85</td>
<td>30K</td>
<td>17.57</td>
<td>1707</td>
<td>249</td>
</tr>
<tr>
<td>tolbutamide - glucose</td>
<td>6.90</td>
<td>30K</td>
<td>21.68</td>
<td>1384</td>
<td>201</td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>6.90</td>
<td>30K</td>
<td>15.86</td>
<td>1892</td>
<td>274</td>
</tr>
<tr>
<td>phenethylbiguanide</td>
<td>6.45</td>
<td>30K</td>
<td>14.67</td>
<td>2045</td>
<td>317</td>
</tr>
<tr>
<td>PEBG - glucose</td>
<td>7.51</td>
<td>30K</td>
<td>15.77</td>
<td>1902</td>
<td>254</td>
</tr>
<tr>
<td>PEBG - glucose</td>
<td>7.10</td>
<td>30K</td>
<td>13.12</td>
<td>2287</td>
<td>322</td>
</tr>
</tbody>
</table>
SUMMARY AND CONCLUSIONS:

The action of the hypoglycemic compounds, tolbutamide and phenethylbiguanide, on protein biosynthesis was investigated by the use of rat liver homogenates, the necessary cofactors and leucine-\(^{14}\)C. The results show that the incorporation of leucine-\(^{14}\)C into hepatic protein is inhibited by both compounds, though in a strikingly different manner. The inhibition by tolbutamide is linear with respect to concentration whereas the inhibition by phenethylbiguanide is not evident until a certain concentration is reached. At this point, the amount of incorporation decreases markedly until it levels off at a very low value. It has been postulated that there is some substance present which combines with phenethylbiguanide and renders it inactive toward inhibition of protein synthesis. Glucose was investigated in this respect and found to have no effect on either tolbutamide or phenethylbiguanide induced inhibition.

The catabolism of leucine was measured by the amount of \(^{14}\)O\(_2\) liberated in the course of the incubation. Both tolbutamide and phenethylbiguanide were found to inhibit \(\mathrm{CO}_2\) production. Again, inhibition by tolbutamide was linear with respect to concentration. Phenethylbiguanide caused a pronounced initial inhibition which tended to level off near the same concentration where the protein inhibition first occurs. This suggests that one of
the reasons that low concentrations of PEBG does not inhibit protein incorporation is due to the great decrease in leucine catabolism. Since less leucine is broken down (catabolized), there is necessarily more present to be incorporated into the protein. According to this postulation, the initial great decrease in leucine catabolism compensates for the inhibition in protein anabolism. Once the inhibition of protein catabolism has levelled off, the inhibition of incorporation by added PEBG becomes evident.

The results found are exactly opposite those reported by Krahl (74) for insulin. Whereas tolbutamide and phenethylbiguanide inhibit protein synthesis in vitro, insulin accelerates it. Moreover, while the accelerating action of insulin is dependent on the presence of glucose in the medium; the inhibitory action of the oral hypoglycemic agents is totally unresponsive to the addition of glucose.

These results suggest that the oral hypoglycemic agents studied, although very proficient at lowering blood sugar, do not possess all the other attributes of insulin. They cannot aid the negative protein balance associated with diabetes; they may, in fact, due to their inhibitory effects, aggravate the condition.
**TABLE XXII**

**EFFICACY OF WASHINGS**

All the washings of the beta-lipoprotein fraction were saved. A 1.0 ml. aliquot of each was plated and counted.

<table>
<thead>
<tr>
<th>Washing (procedure)</th>
<th>Counts</th>
<th>Time (min.)</th>
<th>cpm</th>
<th>Volume of Wash (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>supernatant</td>
<td>30K</td>
<td>0.47</td>
<td>64,000</td>
<td>15.5</td>
</tr>
<tr>
<td>NaCl- MgCl</td>
<td>10K</td>
<td>1.95</td>
<td>5,120</td>
<td>7.0</td>
</tr>
<tr>
<td>reprecipitation 1</td>
<td>1K</td>
<td>3.92</td>
<td>254</td>
<td>7.2</td>
</tr>
<tr>
<td>reprecipitation 2</td>
<td>1K</td>
<td>25.39</td>
<td>39</td>
<td>7.2</td>
</tr>
<tr>
<td>reprecipitation 3</td>
<td>1K</td>
<td>25.03</td>
<td>40</td>
<td>7.2</td>
</tr>
<tr>
<td>TCA 1</td>
<td>1K</td>
<td>60.81</td>
<td>16</td>
<td>5.0</td>
</tr>
<tr>
<td>TCA 2</td>
<td>1K</td>
<td>63.68</td>
<td>16</td>
<td>5.0</td>
</tr>
<tr>
<td>ethanol 1</td>
<td>1K</td>
<td>36.02</td>
<td>28</td>
<td>5.0</td>
</tr>
<tr>
<td>ethanol 2</td>
<td>1K</td>
<td>53.95</td>
<td>19</td>
<td>5.0</td>
</tr>
<tr>
<td>ethanol 3</td>
<td>1K</td>
<td>67.49</td>
<td>15</td>
<td>5.0</td>
</tr>
<tr>
<td>acetone 1</td>
<td>1K</td>
<td>61.60</td>
<td>16</td>
<td>5.0</td>
</tr>
<tr>
<td>acetone 2</td>
<td>1K</td>
<td>58.81</td>
<td>17</td>
<td>5.0</td>
</tr>
<tr>
<td>acetone 3</td>
<td>1K</td>
<td>45.96</td>
<td>22</td>
<td>5.0</td>
</tr>
<tr>
<td>benzene</td>
<td>1K</td>
<td>53.90</td>
<td>19</td>
<td>5.0</td>
</tr>
<tr>
<td>background</td>
<td>1K</td>
<td>53.81</td>
<td>19</td>
<td>-</td>
</tr>
</tbody>
</table>

After the first ethanol wash, essentially a background count is found in the supernatant.
TABLE XXIII

EFFICACY OF WASHINGS

All the washings of the protein fraction were saved. A 1.0 ml. aliquot of each washing was plated and counted.

<table>
<thead>
<tr>
<th>Washing</th>
<th>Counts</th>
<th>Time (min.)</th>
<th>cpm</th>
<th>Volume of Wash (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>supernatant</td>
<td>30K</td>
<td>1.09</td>
<td>27,500</td>
<td>6.2</td>
</tr>
<tr>
<td>TCA</td>
<td>10K</td>
<td>2.13</td>
<td>4,700</td>
<td>5.0</td>
</tr>
<tr>
<td>ethanol 1</td>
<td>3K</td>
<td>2.89</td>
<td>346</td>
<td>5.0</td>
</tr>
<tr>
<td>ethanol 2</td>
<td>1K</td>
<td>6.74</td>
<td>149</td>
<td>5.0</td>
</tr>
<tr>
<td>ethanol 3</td>
<td>1K</td>
<td>33.98</td>
<td>29</td>
<td>5.0</td>
</tr>
<tr>
<td>acetone 1</td>
<td>1K</td>
<td>59.95</td>
<td>17</td>
<td>5.0</td>
</tr>
<tr>
<td>acetone 2</td>
<td>571</td>
<td>39.48</td>
<td>14</td>
<td>5.0</td>
</tr>
<tr>
<td>acetone 3</td>
<td>500</td>
<td>30.17</td>
<td>18</td>
<td>5.0</td>
</tr>
</tbody>
</table>

After the third ethanol wash, a background count is obtained. This indicates that the washings employed were sufficient to remove nonincorporated radioactivity.
TABLE XXIV

EFFECT OF CONCENTRATION OF PHENETHYLBIGUANIDE
ON $^{14}$C$_2$O$_2$ RELEASE (Experiment 10)

<table>
<thead>
<tr>
<th>Umoles of PEBG added</th>
<th>Weight $\text{BaCO}_3$ (mg.)</th>
<th>Counts</th>
<th>Time (min)</th>
<th>cpm</th>
<th>cpm corr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16.8</td>
<td>100K</td>
<td>3.17</td>
<td>31,500</td>
<td>77,700</td>
</tr>
<tr>
<td>0</td>
<td>12.8</td>
<td>100K</td>
<td>2.78</td>
<td>36,000</td>
<td>75,900</td>
</tr>
<tr>
<td>2</td>
<td>10.9</td>
<td>100K</td>
<td>3.73</td>
<td>26,800</td>
<td>50,700</td>
</tr>
<tr>
<td>2</td>
<td>7.5</td>
<td>100K</td>
<td>3.47</td>
<td>28,800</td>
<td>47,100</td>
</tr>
<tr>
<td>4</td>
<td>13.5</td>
<td>100K</td>
<td>5.38</td>
<td>18,600</td>
<td>37,700</td>
</tr>
<tr>
<td>4</td>
<td>10.9</td>
<td>100K</td>
<td>5.04</td>
<td>19,800</td>
<td>36,300</td>
</tr>
<tr>
<td>6</td>
<td>9.9</td>
<td>100K</td>
<td>6.03</td>
<td>16,600</td>
<td>28,600</td>
</tr>
<tr>
<td>6</td>
<td>6.3</td>
<td>100K</td>
<td>4.85</td>
<td>20,600</td>
<td>30,800</td>
</tr>
<tr>
<td>8</td>
<td>10.3</td>
<td>100K</td>
<td>5.54</td>
<td>18,100</td>
<td>31,800</td>
</tr>
<tr>
<td>8</td>
<td>12.4</td>
<td>100K</td>
<td>7.14</td>
<td>14,000</td>
<td>26,300</td>
</tr>
<tr>
<td>10</td>
<td>11.4</td>
<td>100K</td>
<td>7.21</td>
<td>13,900</td>
<td>25,200</td>
</tr>
<tr>
<td>10</td>
<td>7.6</td>
<td>100K</td>
<td>7.84</td>
<td>12,800</td>
<td>19,500</td>
</tr>
<tr>
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<td>10.0</td>
<td>100K</td>
<td>9.31</td>
<td>10,700</td>
<td>17,900</td>
</tr>
<tr>
<td>15</td>
<td>10.8</td>
<td>100K</td>
<td>9.85</td>
<td>10,200</td>
<td>17,600</td>
</tr>
</tbody>
</table>


### TABLE XXV

**EFFECT OF CONCENTRATION OF PHENETHYLBIGUANIDE ON INCORPORATION OF LEUCINE-1-\textsuperscript{14}C INTO PROTEIN OF RAT LIVER (Experiment 10).**

<table>
<thead>
<tr>
<th>Umoles of PEBG added</th>
<th>Weight Protein mg.</th>
<th>Counts</th>
<th>Time (min)</th>
<th>cpm</th>
<th>cpm/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.30</td>
<td>10K</td>
<td>5.78</td>
<td>1730</td>
<td>237</td>
</tr>
<tr>
<td>0</td>
<td>7.00</td>
<td>10K</td>
<td>3.75</td>
<td>2607</td>
<td>381</td>
</tr>
<tr>
<td>2</td>
<td>7.30</td>
<td>10K</td>
<td>5.52</td>
<td>1812</td>
<td>248</td>
</tr>
<tr>
<td>2</td>
<td>7.15</td>
<td>10K</td>
<td>5.50</td>
<td>1818</td>
<td>254</td>
</tr>
<tr>
<td>4</td>
<td>7.00</td>
<td>10K</td>
<td>6.19</td>
<td>1616</td>
<td>231</td>
</tr>
<tr>
<td>4</td>
<td>7.40</td>
<td>10K</td>
<td>6.32</td>
<td>1582</td>
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<td>1511</td>
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<td>7.15</td>
<td>10K</td>
<td>5.77</td>
<td>1733</td>
<td>242</td>
</tr>
<tr>
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<td>7.25</td>
<td>10K</td>
<td>8.31</td>
<td>1203</td>
<td>166</td>
</tr>
<tr>
<td>8</td>
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<td>10K</td>
<td>9.91</td>
<td>1009</td>
<td>142</td>
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<tr>
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<td>10K</td>
<td>11.54</td>
<td>867</td>
<td>126</td>
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<td>10K</td>
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<td>689</td>
<td>94</td>
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<td>5K</td>
<td>11.12</td>
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<tr>
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<td>3K</td>
<td>7.13</td>
<td>421</td>
<td>60</td>
</tr>
</tbody>
</table>
BIBLIOGRAPHY


APPROVAL

The thesis submitted by Lawrence R. DeChatelet has been read and approved by three members of the faculty of Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

[Signature]

Date

Signature of Adviser