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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.

In September 1963, the author began his advanced studies in the Graduate School of Loyola University, Department of Biochemistry. From September 1963, to September 1964, he held the position of Research Assistant in the Department of Biochemistry at the Stritch School of medicine of Loyola University, Chicago, Illinois. The position was supported financially by a grant from the Chicago Heart Association. From September 1964, to the present he was a Cooperative Graduate Fellow of the National Science Foundation, Washington, D.C.

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

ACKNOWLEDGEMENT

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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-C^{14}. The results show that the incorporation of leucine-C^{14} 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 $C^{14}O_2$ 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 (74) for insulin. Whereas tolbutamide and phenethyl-biguanide 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-

1

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 con-
secutive 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 per-
formed 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 a-
meliorated by hypophysectomy (59). The first practical oral hypo-
glycemic 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 inves-
tigators.

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 glucokinase. 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 com-
partment, 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 pos-
sessed an impaired ability to synthesize fat was obtained by
Stetten and Boxer (127) when they demonstrated that the incorpora-
tion of deuterium-labeled water into fatty acids was greatly de-

pressed in the intact diabetic rat. This finding was readily confirmed with C^{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^{14}, as well as from glucose-C^{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 re-
sult of their inability to synthesize fatty acids, and it is evi-
dent 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 nor-
mal 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 C^{14}- labeled tri-
palmitin in diabetic and normal rats, he demonstrated that the
diabetic oxidizes fatty acids to carbon dioxide at a rate consid-
erably above the normal. Further, this increased oxidation of fat
seems clearly to be due to the decreased amount of glucose under-
going 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-C^{14}. 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 C^{14}- glycine or C^{14}-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^{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).

tion of glucose transport.

B. Liver: The work on amino acid incorporation in liver slices has been performed by Krahl (74). 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 accompa-
nying disturbances in lipid metabolism rather than to the so
called "primary" disturbance in carbohydrate metabolism. If dia-
betes mellitus is to be successfully controlled, all of the accom-
panying 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 hypo-
glycemic agents should be effective in reducing the hypercholes-
terolemia 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 A11, 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          Phenylalanine
            Isoleucine         Valine
              Valine           Asparagine
          Glutamic acid        Glutamine
            Glutamine          Histidine
S ——————————— Cysteine         Leucine
            Cysteine— S - S— Cysteine
             Alanine           Glycine
              Serine           Serine
              Valine           Histidine
S ————————————— Cysteine       Leucine
              Serine           Valine
             Leucine           Glutamic acid
             Tyrosine          Alanine
            Glutamine          Leucine
             Leucine           Tyrosine
          Glutamic acid
            Asparagine         Leucine
             Tyrosine          Valine
          Cysteine—S - S—Cysteine
            Asparagine         Glycine
                               Glutamic acid
                               Arginine
             A CHAIN           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 amino-
peptidase which can attack the insulin structure only at the amino
ends of the chains. From this work, it was concluded that the
amino acids B1-B6 were not necessary for full hormonal activity.
However, the removal of A1-A5 probably leads to some loss of ac-
tivity.

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 physiolog-
ical 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 exper-
imental findings pertinent to the structure of insulin will be
briefly mentioned.

Insulin has been labeled by esterification of the ali-
phatic 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 compounds which could be administered orally has long been in effect. As early as 1926, Frank, Nothman, and Wagner (45) reported a hypoglycemic 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 substitute.

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, lapanine. A wide range of natural sources have yielded compounds with hypoglycemic properties. 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, <u>Blighia</u> <u>sapida</u>. 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

H_2N- [benzene ring] $C - SO_2NHCONH(CH_2)_3CH_3$ Carbutamide

H_3C- [benzene ring] $C - SO_2NHCONH(CH_2)_3CH_3$ Tolbutamide

$Cl-$ [benzene ring] $C - SO_2NHCONH(CH_2)_2CH_3$ Chlorpropamide

H_3C- [benzene ring with NH_2 substituent] $C - SO_2NHCONH - C$ [benzene ring]   Metahexamide
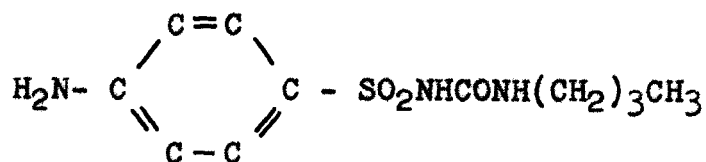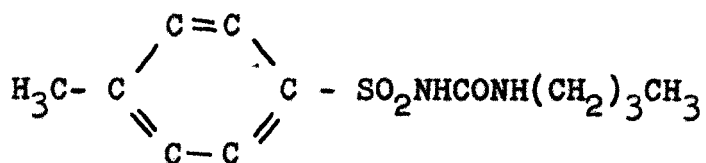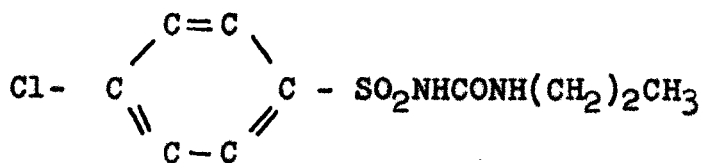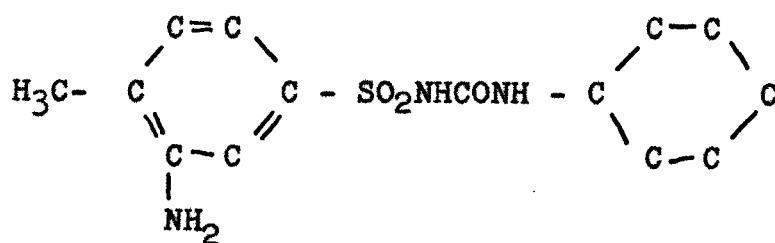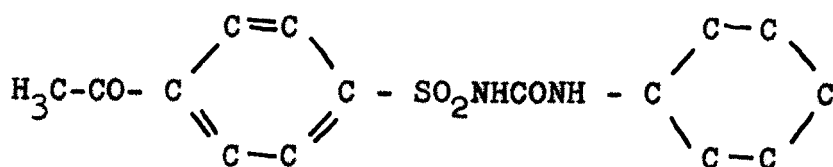
H_3C-CO- [benzene ring] $C - SO_2NHCONH - C$ [cyclohexane ring]   Acetohexamide

TABLE III

GUANIDINE AND SOME DERIVATIVES

$H_2N - C - NH_2$
$\quad\quad \| $
$\quad\quad NH$
Guanidine
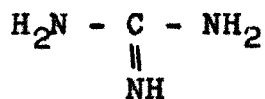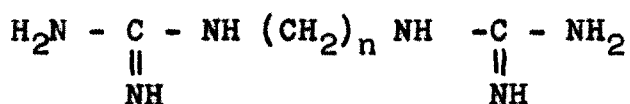
$H_2N - C - NH\ (CH_2)_n\ NH\ -C - NH_2$
$\quad\quad \|\quad\quad\quad\quad\quad\quad\quad \|$
$\quad\quad NH\quad\quad\quad\quad\quad\quad\quad NH$
Synthalin A (n = 10)

Synthalin B (n = 12)

$C - (CH_2)_2\ NHCNHCNH_2$
$\quad\quad\quad\quad\quad\quad \|\ \ \|$
$\quad\quad\quad\quad\quad\quad NH\ NH$
Phenethylbiguanide

$H_3C - C - C - CH_2CHCOO^-$
$\quad\quad \backslash / \quad\quad\quad |$
$\quad\quad C\quad\quad\quad\quad NH_3$
Hypoglycin A

$H_3C - C - C - CHNHCO(CH_2)_2CHCOOH$
$\quad\quad \backslash / \quad\quad |\quad\quad\quad\quad\quad |$
$\quad\quad C\quad\quad COOH\quad\quad\quad NH_2$
Hypoglycin B
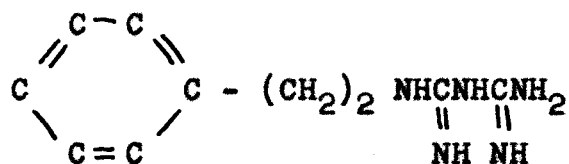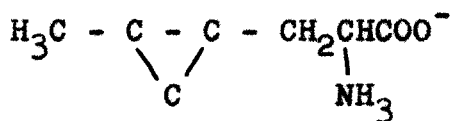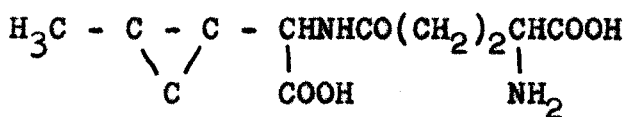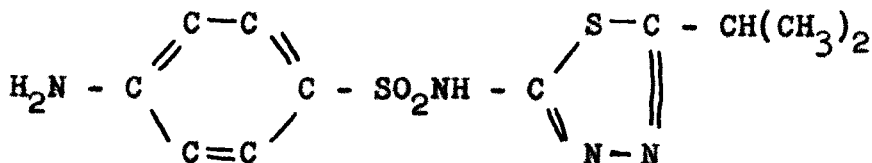
$H_2N - C$ ... $C - SO_2NH - C$ ... $S-C - CH(CH_3)_2$ ... $N-N$
IPTD

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 glu-
cose administration. Chen, Anderson, and Maze (29) have reported
a hypoglycemic action of 5-cyclopropyl-2-sulfanilamide-1,3,4-thia-
diazole in intact rabbits. However, since the thiadiazoles were
very toxic they were never put to clinical use.

About this time, the studies of Franke, Achelis, Betram
et al. (2,14,44) on a new hypoglycemic compound, 1-butyl-3-sulfo-
nylurea (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 hypo-
glycemic 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 pro-
duction 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 I^{131} 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
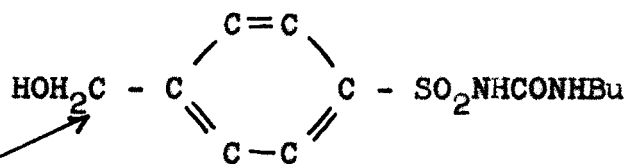
p-tolylsulfonylurea

tolbutamide

p-toluenesulfonamide

N-butyl-N'-p-hydroxymethyl-phenylsulfonylurea
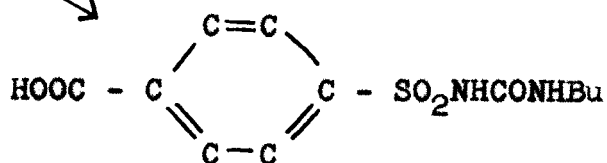
N-butyl-N'-p-carboxy-phenylsulfonylurea
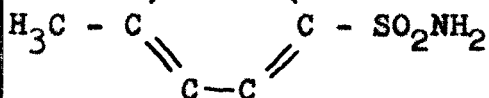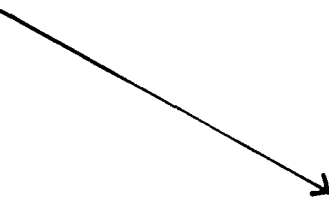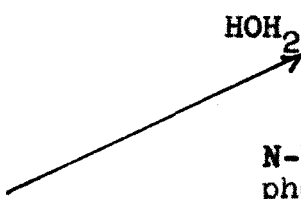
approximately ten times that of tolbutamide (129). Evidence in-
dicates that this compound, in contrast to tolbutamide, is proba-
bly not metabolized to any appreciable extent before excretion
through the kidneys. No definite chromatographic or ultraviolet
absorption differences have been found between the pure drug and
that in serum or urine (68,128). There is, however, a considera-
ble difference between various species of animals. Using the
urine of dogs treated with chlorpropamide-S^{35}, three different
chromatographic spots were detected. They were identified as the
unchanged compound, p-chlorobenzenesulfonylurea, and p-chloroben-
zenesulfonamide. The three compounds accounted for 30, 40, and
20 per cent respectively (68). This serves to illustrate the dan-
ger involved in the indiscriminate application of animal experi-
ments to man. It is always necessary to take into account the
possibility of interspecial 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 in-
creased 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 arylsulfonyl-
ureas 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) demon-
strated 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 hypoglyce-
mic 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 pancreatectomized 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 glu-
cose, acetate and succinate and considerably reduces fat synthe-
sis. In man, the blood pyruvate and lactate levels are raised
(39) and there is no change in hepatic vein glucose, urea, pyru-
vate 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 in-
tact animals a decrease in the blood sugar concentration is easi-
ly produced. These results suggest that the effects of phenethyl-
biguanide are largely mediated in the peripheral tissues.

The tissue anoxia suggested by this evidence may be
attributed to inhibition of cytochrome oxidase and succinic dehy-
drogenase (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 $37^{o}C$ for two hours together with a number of cofactors, an oral hypoglycemic agent, and leucine-1-C^{14}.

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 O.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. Alied 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^{14} and Leucine - C^{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 uc / 50 ul of solution. The aqueous solutions were stored in the freezer at - 5°C and thawed immediately before use.

The L - leucine- 1- C^{14} is especially valuable for studies in CO_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- C^{14} (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 K_2HPO_4 and 1.815 g. of KH_2PO_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 IV

COMPOSITION OF PREMIX

SUBSTANCE	MOLARITY	CONC. g/50 ml.
KCl	0.075	0.280
$MgCl_2 \cdot 6H_2O$	0.01	0.102
$KHCO_3$	0.015	0.076
Glucose	0.015	0.135
Nicotinamide	0.02	0.122
EDTA	0.0003	0.005
NAD	0.0025	0.092
AMP	0.0020	0.045
ATP	0.0020	0.062

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 $0^{o}C$ during the entire homogenization process. Unbroken cells, tissue debris and nuclei were removed by centrifugation at 700g. for 15 min. at $0^{o}C$. 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 $MgCl_2$ 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 MgCl 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 $MgCl_2$ 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 $MgCl_2$ 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 $MgCl_2$.   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 radio-activity 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 benzene 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 smaple 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- C^{14} 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 pricipitate dissolved in 2.0 ml. of O. 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 0.8 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 V

STANDARD CURVE DATA FOR PROTEIN DETERMINATION

mg Protein	Klett Units	Average	Standard Deviation
0.5 0.5 0.5	16.0 14.0 15.0	15.0	1.0
1.0 1.0 1.0	28.5 31.0 30.5	30.0	1.3
1.5 1.5 1.5	45.5 44.0 44.5	44.7	0.8
2.0 2.0 2.0	60.0 64.0 61.0	61.7	2.1
2.5 2.5 2.5	77.0 78.5 76.0	77.2	1.3
3.0 3.0 3.0	92.0 92.5 91.0	91.8	0.8
4.0 4.0 4.0	123.0 119.0 121.5	121.2	2.0
5.0 5.0 5.0	152.0 150.0 154.0	152.0	2.0

Figure III

$C^{14}O_2$ Collection and Measurement

Incubations in which the amount of $C^{14}O_2$ 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_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_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 $BaCl_2$.

The precipitate of $BaCO_3$ which formed was filtered on pre-weighed Whatman # 50 filter paper using a Tracerlab Precipitation Apparatus, Model E8-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)

$$\frac{I}{I_o} = \frac{1-C^{-uh}}{uh}$$

where I = observed activity, I_o = actual activity, u = 0.29 and h = 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 C^{14} source with each group of samples.
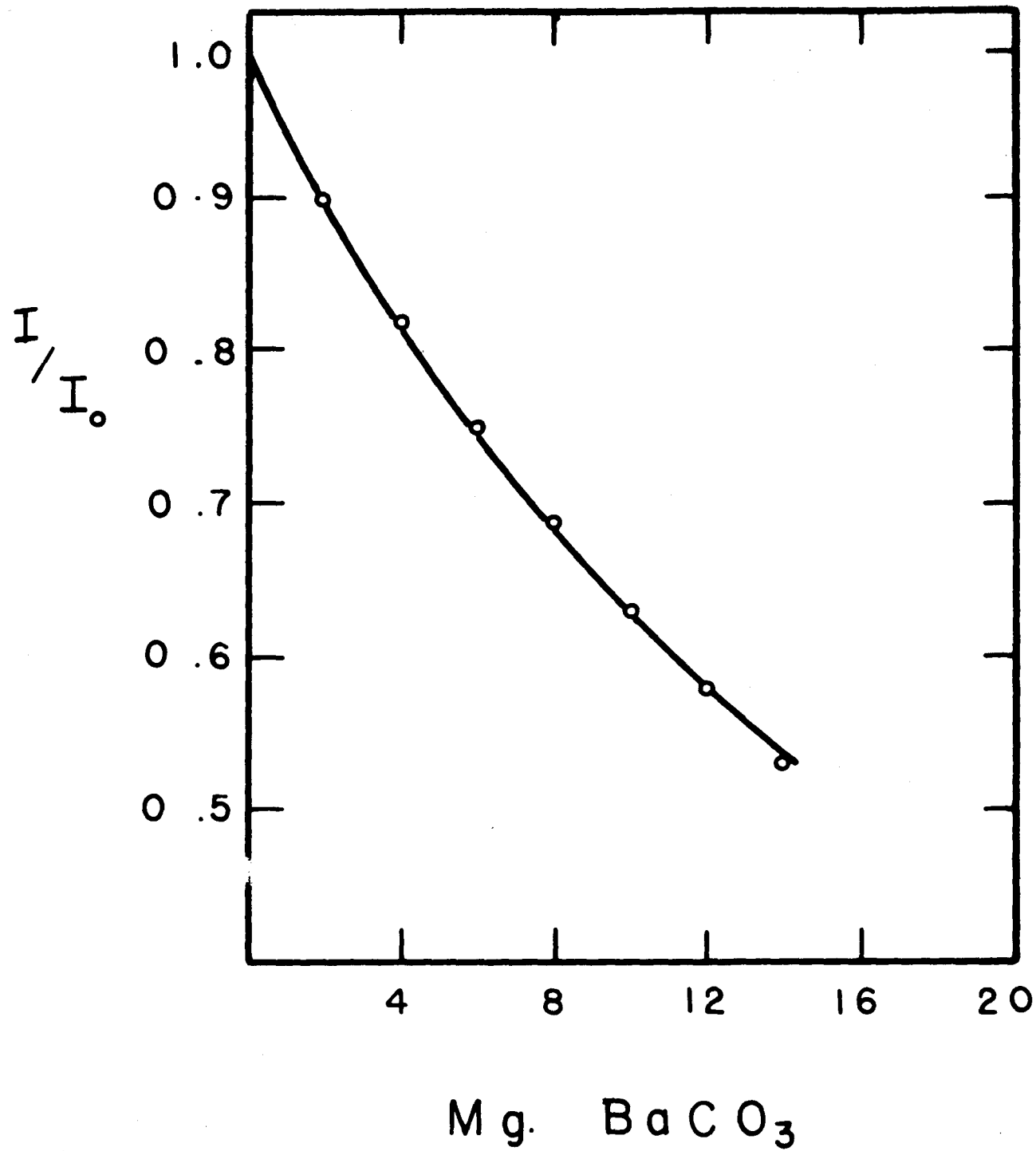
Mg. BaCO₃

Figure IV

TABLE VI

SELF-ABSORPTION CORRECTIONS FOR BaCO$_3$

mg BaCO$_3$	I/I$_o$	mg BaCO$_3$	I/I$_o$
0.0-0.4	1.00	6.7-6.8	0.72
0.5-0.6	0.98	6.9-7.2	0.71
0.7	0.95	7.3-7.7	0.70
0.8-0.9	0.94	7.8-8.0	0.69
1.0-1.5	0.93	8.1-8.3	0.68
1.6-1.7	0.92	8.4-8.6	0.67
1.8-1.9	0.91	8.7-8.9	0.66
2.0-2.1	0.90	9.0-9.3	0.65
2.2-2.3	0.89	9.4-9.6	0.64
2.4-2.6	0.88	9.7-10.0	0.63
2.7-2.9	0.87	10.1-10.4	0.62
3.0-3.1	0.86	10.5-10.8	0.61
3.2-3.4	0.85	10.9-11.2	0.60
3.5	0.84	11.3-11.6	0.59
3.6-3.8	0.83	11.7-12.1	0.58
3.9-4.1	0.82	12.2-12.4	0.57
4.2-4.4	0.81	12.5-12.9	0.56
4.5	0.80	13.0-13.3	0.55
4.6-4.9	0.79	13.4-13.8	0.54
5.0-5.2	0.78	13.9-14.1	0.53
5.3-5.4	0.77	15.0	0.51
5.5-5.7	0.76	15.5-15.8	0.50
5.8-6.0	0.75	16.0	0.49
6.1-6.3	0.74	17.0	0.47
6.4-6.6	0.73	20.0	0.43

CHAPTER IV

EXPERIMENTAL RESULTS

<u>Experiment 1</u>:   <u>Change in pH During the Course of the Incubation</u>.

Before any actual incubations were performed, it was de-
cided 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 ini-
tial 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 (4×10^{-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 val-
ue, so the incubation was repeated using a 3:1 v/w homogenate.
Again the initial and final pH was measured and the results tabu-
lated.   Since the pH change in this case was considerably smaller,

TABLE VII

THE pH OF THE INCUBATION MIXTURES

I. pH of Constituents of Mixtures

pH buffer	7.42
pH premix	7.33
pH homogenate (2:1)	7.04
pH homogenate (3:1)	7.23

II. pH of Incubation Mixtures Using 2:1 Homogenates

Addition	pH start	pH finish
Tolbutamide	7.04	6.86
Chlorpropamide	7.02	6.82
Phenethylbiguanide	7.02	6.59

III. pH of Incubation Mixtures Using 3:1 Homogenates

Addition	pH start	pH finish
Tolbutamide	7.23	7.02
Chlorpropamide	7.21	7.01
Phenethylbiguanide	7.23	6.80

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

Date	Type	pH
8/11/64	rat liver	7.23
8/13/64	rat liver	7.08
8/14/64	rat liver	7.18
8/19/64	dog liver	7.18
9/14/64	rat liver	7.18
10/15/64	rat (2) liver	7.22
11/ 4/64	rat liver	7.22

it was decided that the 3:1 homogenate would be used in all suc-
ceeding 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 un-
dergoes some reaction, peculiar to that agent alone, which re-
leases 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- C^{14} into Beta-Lipo-
protein: Effect of Differing Isotope.

Incubations were run in duplicate at $37^{\circ}C$. for a period
of two hours. One incubation flask was kept at $0^{\circ}C$. to serve as a
control. Radioactivity present in the lipoprotein above that
present in the $0^{\circ}C$ flask was interpreted as representing incorpo-
ration. The incubations were run using 2 uc of either leucine-1-
C^{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 a-
mount of incorporation occurred using the isotope of lower specif-
ic activity. A more significant amount of incorporation was

TABLE VIII

INCORPORATION OF LEUCINE INTO BETA-LIPOPROTEIN:
EFFECT OF SPECIFIC ACTIVITY OF ISOTOPE.

Incub. temp	Leucine-1-C^{14} (sp. act. 25mc/mm)	Leucine-C^{14} (U.L.) (sp. act. 233mc/mm)
$37^{\circ}C$	36 cpm	112 cpm
$37^{\circ}C$	32 cpm	69 cpm
$37^{\circ}C$	30 cpm	84 cpm
$0^{\circ}C$	21 cpm	21 cpm
$0^{\circ}C$	22 cpm	25 cpm
background	20 cpm	18 cpm

evident when the leucine with the higher specific activity was used, but the results were not very reproducible.

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

Each incubation flask contained 2 uc of leucine - C^{14} (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 4×10^{-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

Experimental Procedure	cpm
37° incubation	80
37° incubation	50
Tolbutamide added	58
Tolbutamide added	35
Premix omitted	30
Premix omitted	29
0° control	19
Background	20

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 4×10^{-3} M tolbutamide, and 1 containing 4×10^{-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 $37^{\circ}C$, 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 pre-
cipitated for protein by the addition of 4.0 ml. of 5% TCA,
washed and counted.

Carrier plasma was added to the remainder of the super-
natant 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 sepa-
ration 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, tolbu-
tamide inhibits the incorporation into both fractions to approxi-
mately the same extent. The presence of orotic acid has no ef-
fect 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 tolbu-
tamide 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

Supernatant (10.0 ml)

5% TCA

Divide in two parts

particulate protein ppt.

1.0 ml.

9.0 ml.

4.0 ml. 5% TCA

Add 5.0 ml. Carrier serum

soluble prot. ppt.

Phosphotungstate

beta-lipoprotein ppt.
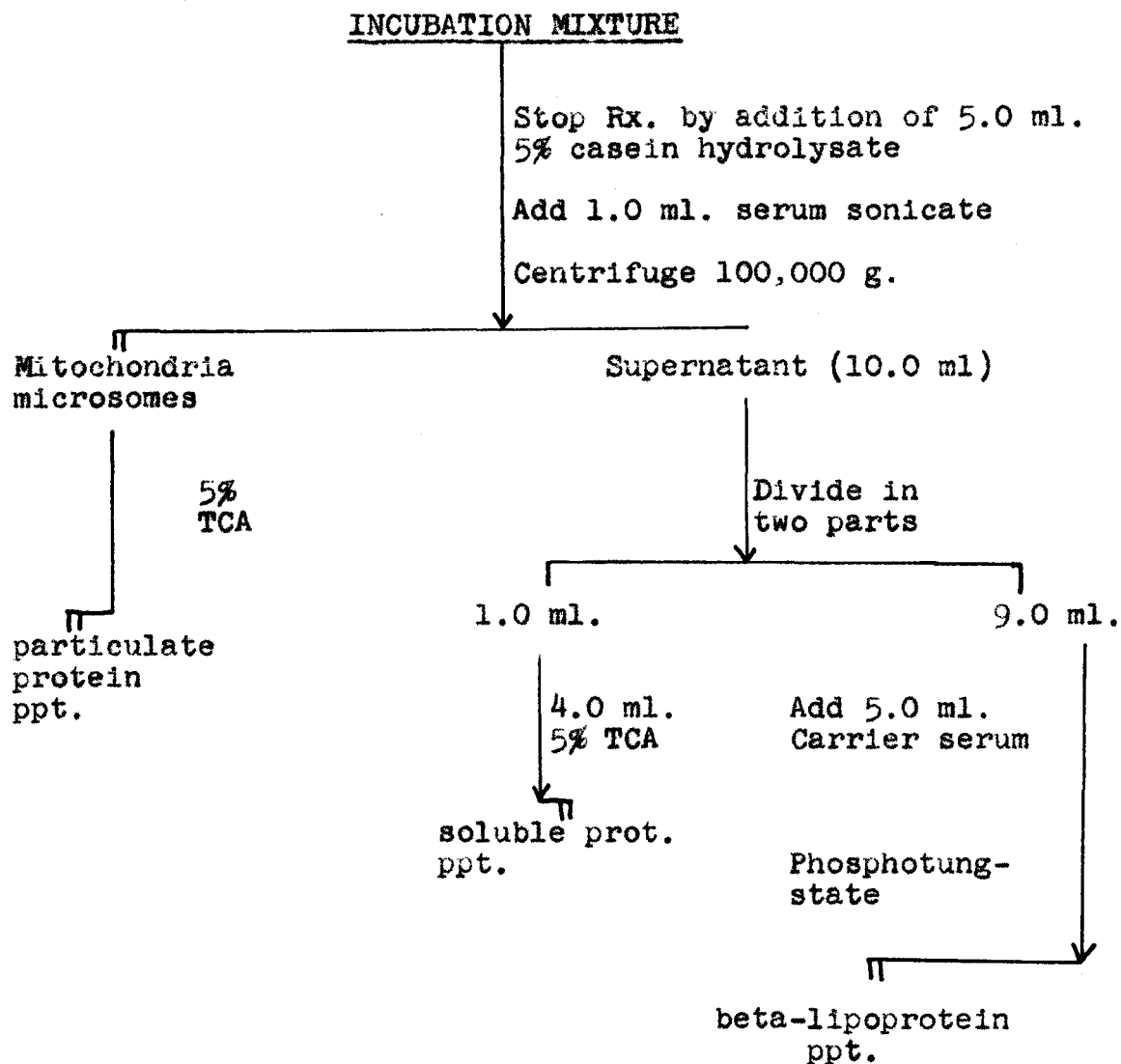
TABLE X

DISTRIBUTION OF RADIOACTIVITY

Fraction	Weight mg.	Counts	Time (min)	cpm	cpm/mg
Protein-soluble					
no addition	4.8	3000	7.22	416	87
no addition	4.6	3000	6.62	453	98
tolbutamide	4.0	3000	11.64	258	65
orotic acid	4.8	3000	7.14	420	88
Protein-particulate					
no addition	9.0	3000	0.79	3797	422
no addition	8.7	3000	0.99	3030	348
tolbutamide	10.1	3000	1.41	2128	211
orotic acid	9.1	3000	0.96	3125	343
Beta-lipoprotein					
no addition	–	3000	31.12	96	–
no addition	–	3000	34.13	88	–
tolbutamide	–	3000	54.81	55	–
orotic acid	–	3000	85.47	35	–

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 CO_2 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 - 1 C^{14} was employed, so that there would be a strict one-to-one correspondence between the amount of leucine catabolized and the amount of $C^{14}O_2$ produced.

TABLE XI

DISTRIBUTION OF RADIOACTIVITY

INCUBATION WITH TOLBUTAMIDE AND PHENETHYLBIGUANIDE

Fraction	Weight mg.	Counts	Time (min)	cpm	cpm/mg
Protein-soluble					
no addition	11.0	3000	3.18	943	86
no addition	11.0	3000	2.62	1145	104
tolbutamide	9.8	3000	5.07	592	60
tolbutamide	9.3	3000	5.29	567	61
phenethylbiguanide	9.6	3000	3.27	917	96
phenethylbiguanide	10.3	3000	3.03	990	96
Protein-particulate					
no addition	5.3	10000	1.97	5076	958
no addition	6.0	10000	1.85	5368	964
tolbutamide	6.2	10000	2.71	3690	595
tolbutamide	5.9	10000	2.57	3891	659
phenethylbiguanide	5.2	10000	1.85	5405	1039
phenethylbiguanide	5.8	10000	1.57	6369	1098
Beta-lipoprotein					
no addition	8.3	3000	7.62	394	47
no addition	7.8	3000	8.97	334	43
tolbutamide	4.0	3000	18.64	161	40
tolbutamide	9.9	3000	8.57	350	35
phenethylbiguanide	7.0	3000	8.99	334	48
phenethylbiguanide	11.2	3000	5.50	545	49

TABLE XII

SUMMARY OF EXPERIMENTS 4 AND 5

Fraction	Experiment 4		Experiment 5	
	Average sp. act.	% of Control	Average sp. act.	% of Control
Protein-soluble				
Control	93	100	95	100
tolbutamide	65	70	60	63
phenethylbiguanide	-	-	96	100
Protein-particulate				
Control	385	100	961	100
tolbutamide	211	55	528	70
phenethylbiguanide	-	-	1003	105
Beta-lipoprotein				
control	-	-	45	100
tolbutamide	-	-	38	85
phenethylbiguanide	-	-	48	106

FIGURE VI

THE CATABOLISM OF LEUCINE (137)
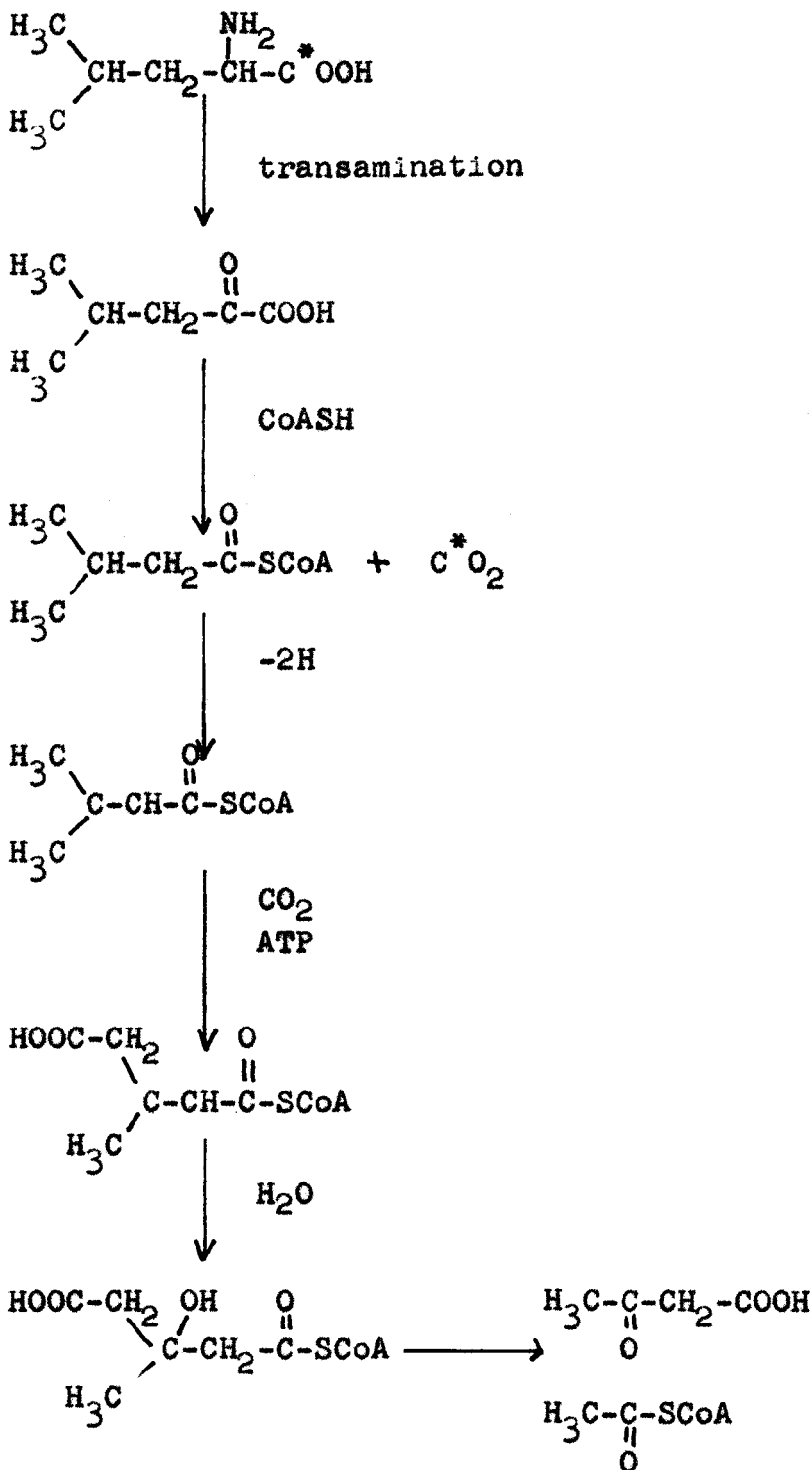
Experiment 6: Effect of Tolbutamide and Phenethylbiguanide on $C^{14}O_2$ Production from Leucine - 1 - C^{14}.

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 pre- cipitate 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 spe- cific 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

CO_2 RELEASE AND PROTEIN INCORPORATION FROM EXPERIMENT 6

CO_2 DATA (CATABOLISM)

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

PROTEIN INCORPORATION (ANABOLISM)

Sample	Weight mg.	Counts	Time (min)	cpm	cpm/mg
control	4.8	10K	7.56	1320	276
tolbutamide	4.6	10K	18.38	543	118
tolbutamide	5.0	10K	14.64	682	136
phenethylbiguanide	5.1	10K	9.97	1000	196
phenethylbiguanide	4.8	10K	9.10	1100	229

considered. Substance x depresses the catabolism of leucine only and has no effect on other sources of CO_2. Substance y depresses the catabolism of leucine but also depresses CO_2 production via the Krebs cycle.

	cpm	CO_2 from leucine	Other CO_2	cpm/mg
control	100	5 mg	5 mg	10
x added	25	1.25 mg	5 mg	4
y added	25	1.25 mg	1.25 mg	10

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 CO_2 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.

$$\frac{catabolism}{anabolism} = \frac{51,500}{1320} = 39$$

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 CO_2 release. The incubation flasks contained 0.5 ml. of homogenate, 0.5 ml. of premix, 1 uc of leucine-1-C^{14}, and sufficient oral hypoglycemic agent to make the final concentration 4×10^{-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 4×10^{-3} M tolbutamide, chlorpropamide and phenethylbiguanide. Data are presented in Table XIV.

The data on the release of $C^{14}O_2$ 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

CO_2 DATA

Sample	Weight mg.	Counts	Time (min)	cpm	cpm corr.
no addition	8.1	100K	8.70	11,494	17,749
tolbutamide	5.6	100K	8.25	12,121	16,904
tolbutamide	7.5	100K	9.36	10,684	16,091
Chlorpropamide	5.9	100K	12.50	8,000	11,093
Chlorpropamide	5.8	100K	12.52	7,987	11,076
phenethylbiguanide	7.3	100K	12.74	7,849	11,641
phenethylbiguanide	8.3	100K	12.83	7,794	11,902
kill (TCA)	11.3	2352	23.50	100	169

PROTEIN DATA

Sample	Weight mg.	Counts	Time (min)	cpm	cpm/mg
no addition	7.1	10K	60.34	166	23
tolbutamide	5.9	10K	47.56	210	36
tolbutamide	5.5	10K	58.72	170	31
chlorpropamide	5.8	10K	54.48	184	32
chlorpropamide	5.7	10K	52.97	189	33
phenethylbiguanide	6.1	10K	73.14	137	22
phenethylbiguanide	5.7	10K	81.68	122	21
kill (TCA)	5.3	5224	290.18	18	3

effect very similar to phenethylbiguanide. This last observation
is most surprising, since chlorpropamide is very similar struc-
turally to tolbutamide and totally unlike phenethylbiguanide.

The protein data from this experiment are inconclusive,
since the specific activities obtained are too low to be meaning-
ful. This is evidently a result of the dilution of the incuba-
tion mixture (it was one-half as concentrated as in previous ex-
periments) and the fact that only 1 uc. of leucine-1-C^{14} was add-
ed to each flask.

Experiment 8: The effect of the Concentration of Phenethyl-biguanide on $C^{14}O_2$ 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^{14}. Added to this was a varying amount
of phenethylbiguanide (from 0-30 umoles) dissolved in 1.0 ml. of
H_2O, so that the total volume of each flask was 2.0 ml. The radio-
activity present in the CO_2 collected is given in Table XV.

The inhibition of CO production is illustrated graphic-
ally in Figure VII.

TABLE XV

EFFECT OF CONCENTRATION OF PHENETHYL-
BIGUANIDE ON $C^{14}O_2$ EVOLUTION (Experiment 8)

Umoles of PEBG added	Weight BaCO$_3$(mg.)	Counts	Time (min)	cpm	cpm corr.
0	9.3	30K	5.52	5440	8590
0	9.3	30K	5.07	5930	9400
4	8.2	30K	6.45	4650	6960
4	7.0	30K	6.06	4945	7140
8	16.2	30K	10.76	2785	5770
8	10.7	30K	8.04	3735	6240
12	6.6	30K	8.46	3542	4950
12	8.9	30K	9.24	3250	5000
16	10.4	30K	10.22	2935	4810
16	13.8	30K	11.62	2580	4850
20	6.6	30K	9.64	3115	4340
30	9.2	10K	3.58	2795	4360
30	7.9	10K	3.51	2850	4180
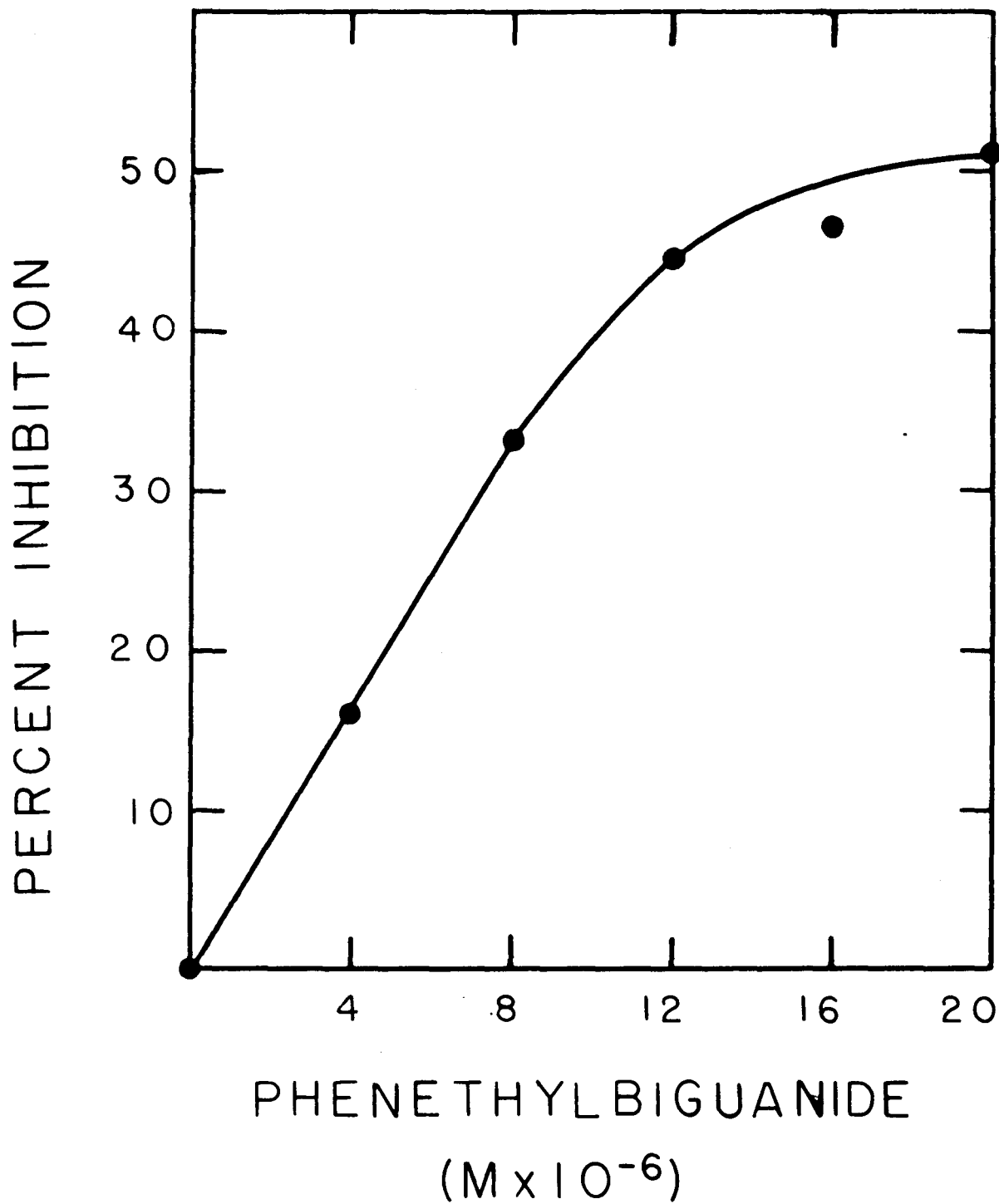
Figure VII

Experiments 9 & 10: The Effect of the Concentration of Phenethyl-biguanide on $C^{14}O_2$ Evolution and Protein Incorporation.

Incubations were performed using 0.5 ml. of homogenate, 0.5 ml. of premix, 2 ucuries of leucine- 1- C^{14}, and varying a-mounts of phenethylbiguanide. The total volume in each flask was 1.0 ml. Evolved 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 exper-iment, and the results are presented in the appendices in Tables XXIV and XXV.

Experiment 11: The Effect of the Concentration of Tolbutamide on $C^{14}O_2$ Evolution and Protein Incorporation.

The experiment was performed exactly as the preceding two, except that tolbutamide was substituted for the phenethyl-biguanide. Both protein and 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 CO_2 production and protein synthesis is presented in Figures VIII

TABLE XVI

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

Umoles of PEBG added	Weight $BaCO_3$(mg.)	Counts	Time (min)	cpm	cpm corr.
0	14.8	100K	4.60	21,739	47,306
0	15.3	100K	4.57	21,882	48,582
2	13.9	100K	7.79	12,837	25,815
2	11.8	100K	7.04	14,204	26,190
4	12.6	100K	8.70	11,494	21,786
4	12.8	100K	8.79	11,377	21,571
6	16.5	100K	12.18	8,210	17,771
8	11.6	100K	10.68	9,363	16,762
8	9.9	100K	9.82	10,183	16,957
10	16.8	100K	12.84	7,788	17,251
10	19.9	100K	15.57	6,423	15,356
15	13.5	100K	14.75	6,779	12,963
15	13.0	100K	12.47	8,019	15,162

TABLE XVII

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

Umoles of PEBG added	Weight Protein mg.	Counts	Time (min)	cpm	cpm/mg
0	7.45	10K	6.70	1493	200
0	7.65	10K	5.59	1789	234
2	6.85	10K	7.47	1339	195
4	6.95	10K	8.33	1200	173
4	7.15	10K	7.41	1350	189
6	6.95	10K	10.33	968	139
6	6.65	10K	8.19	1221	184
8	7.00	10K	18.64	536	77
8	6.65	10K	14.33	698	105
10	6.80	10K	33.79	296	44
10	7.15	10K	49.42	202	28
15	7.10	10K	50.43	198	28
15	7.05	10K	50.11	200	28

TABLE XVIII

EFFECT OF CONCENTRATION OF TOLBUTAMIDE
ON $C^{14}O_2$ EVOLUTION

Umoles of Tolbutamide added	Weight $BaCO_3$(mg.)	Counts	Time (min)	cpm	cpm corr.
0	12.5	100K	3.42	29,200	60,000
0	11.7	100K	3.25	30,700	61,100
2	10.8	100K	3.52	28,400	53,100
2	10.3	100K	3.18	31,400	58,500
4	13.2	100K	3.96	25,300	51,800
4	12.5	100K	3.66	27,300	55,300
6	11.3	100K	3.97	25,200	48,100
6	11.0	100K	4.20	23,800	44,400
8	14.1	100K	4.91	20,400	42,300
8	15.9	100K	5.22	19,200	42,900
10	13.9	100K	5.25	19,000	39,300
10	7.3	100K	4.43	22,600	36,000
15	6.6	100K	4.04	24,800	38,200
15	11.7	100K	4.55	22,000	42,100

TABLE XIX

EFFECT OF CONCENTRATION OF TOLBUTAMIDE

ON INCORPORATION OF LEUCINE-1-C^{14} INTO

PROTEIN OF RAT LIVER

| Umoles of tolbutamide added	Weight Protein mg.	Counts	Time (min)	cpm	cpm/mg
0	8.60	10K	4.85	2062	238
0	8.45	10K	4.44	2252	267
2	8.35	10K	5.94	1684	202
2	8.45	10K	5.02	1992	236
4	8.20	10K	5.62	1779	217
4	8.00	10K	5.68	1760	220
6	8.35	10K	6.81	1468	176
6	7.80	10K	7.11	1406	180
8	7.65	10K	8.41	1189	155
8	7.95	10K	9.81	1019	128
10	7.60	10K	8.46	1182	156
10	7.80	10K	7.45	1342	172
15	8.10	10K	9.14	1094	135
15	8.70	10K	10.82	924	106
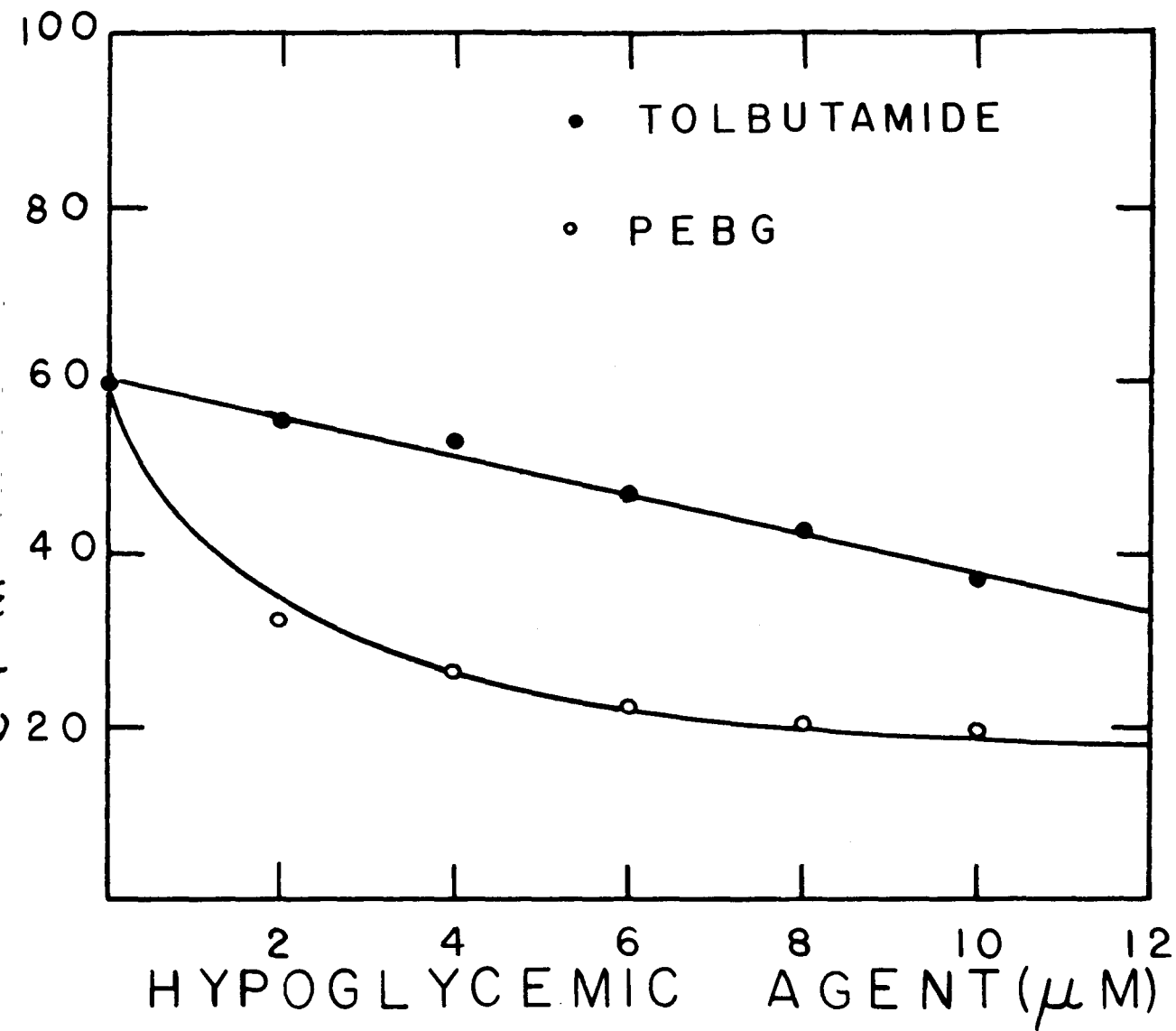
Figure VIII
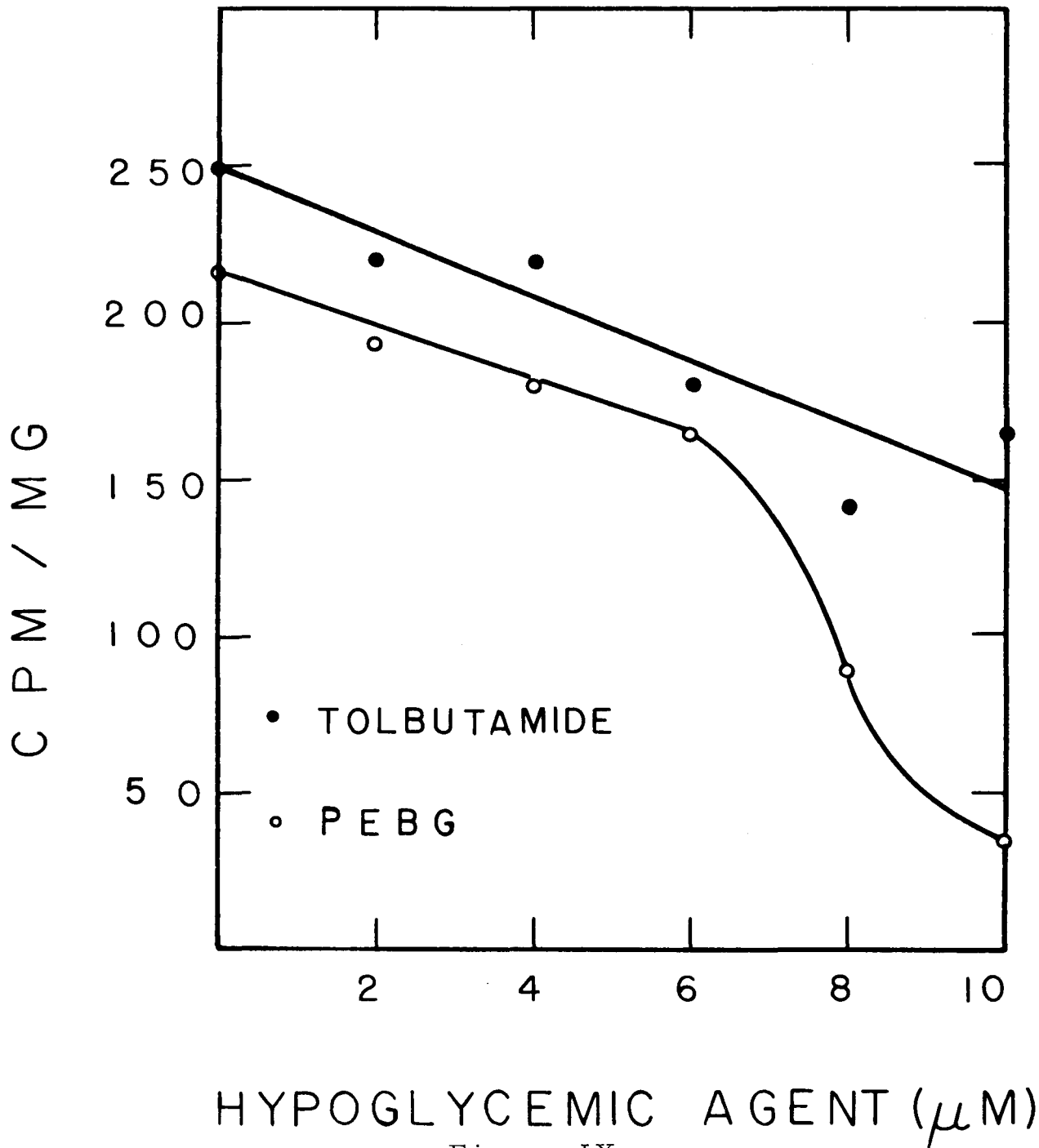
Figure IX

and 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-C^{14} 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.

<u>Experiment 12</u>: <u>The</u> <u>Effect</u> <u>of</u> <u>Glucose</u> <u>on</u> <u>Inhibition</u> <u>by</u> <u>Hypoglycemic</u> <u>Agents</u> <u>of</u> <u>Leucine</u> <u>Anabolism</u> <u>and</u> <u>Catabolism</u>.

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 $C^{14}O_2$ RELEASE

Incubation	Weight $BaCO_3$ mg.	Counts	Time (min)	cpm	cpm corr.
control	11.5	100K	13.46	7429	12,592
control	13.1	100K	14.26	7013	12,751
control - glucose	9.7	100K	13.21	7570	12,159
control - glucose	9.2	100K	11.89	8410	12,938
tolbutamide	9.8	100K	15.22	6570	10,429
tolbutamide	10.0	100K	15.06	6640	10,540
tolbutamide - glucose	7.7	100K	15.00	6666	9,523
tolbutamide - glucose	10.7	100K	17.01	5879	9,638
phenethylbiguanide	8.6	100K	32.69	3059	4,566
phenethylbiguanide	8.3	100K	29.53	3386	4,979
PEBG - glucose	8.1	100K	31.05	3221	4,737

TABLE XXI

EFFECT OF GLUCOSE AND ORAL HYPOGLYCEMIC AGENTS

ON INCORPORATION OF LEUCINE-C^{14} INTO PROTEIN

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

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-C^{14}. The results show that the incorporation of leucine-C^{14} 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 $C^{14}O_2$ 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 (74) for insulin. Whereas tolbutamide and phenethyl-biguanide 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.

A P P E N D I X

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.

Washing (procedure)	Counts	Time (min.)	cpm	Volume of Wash(ml.)
supernatant	30K	0.47	64,000	15.5
NaCl- MgCl	10K	1.95	5,120	7.0
reprecipitation 1	1K	3.92	254	7.2
reprecipitation 2	1K	25.39	39	7.2
reprecipitation 3	1K	25.03	40	7.2
TCA 1	1K	60.81	16	5.0
TCA 2	1K	63.86	16	5.0
ethanol 1	1K	36.02	28	5.0
ethanol 2	1K	53.95	19	5.0
ethanol 3	1K	67.49	15	5.0
acetone 1	1K	61.60	16	5.0
acetone 2	1K	58.81	17	5.0
acetone 3	1K	45.96	22	5.0
benzene	1K	53.90	19	5.0
background	1K	53.81	19	-

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.

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

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 $C^{14}O_2$ RELEASE (Experiment 10)

Umoles of PEBG added	Weight $BaCO_3$ (mg.)	Counts	Time (min)	cpm	cpm corr.
0	16.8	100K	3.17	31,500	77,700
0	12.8	100K	2.78	36,000	75,900
2	10.9	100K	3.73	26,800	50,700
2	7.5	100K	3.47	28,800	47,100
4	13.5	100K	5.38	18,600	37,700
4	10.9	100K	5.04	19,800	36,300
6	9.9	100K	6.03	16,600	28,600
6	6.3	100K	4.85	20,600	30,800
8	10.3	100K	5.54	18,100	31,800
8	12.4	100K	7.14	14,000	26,300
10	11.4	100K	7.21	13,900	25,200
10	7.6	100K	7.84	12,800	19,500
15	10.0	100K	9.31	10,700	17,900
15	10.8	100K	9.85	10,200	17,600

TABLE XXV

EFFECT OF CONCENTRATION OF PHENETHYLBIGUANIDE ON

INCORPORATION OF LEUCINE-1-C^{14} INTO

PROTEIN OF RAT LIVER (Experiment 10).

Umoles of PEBG added	Weight Protein mg.	Counts	Time (min)	cpm	cpm/mg
0	7.30	10K	5.78	1730	237
0	7.00	10K	3.75	2667	381
2	7.30	10K	5.52	1812	248
2	7.15	10K	5.50	1818	254
4	7.00	10K	6.19	1616	231
4	7.40	10K	6.32	1582	214
6	7.30	10K	6.62	1511	207
6	7.15	10K	5.77	1733	242
8	7.25	10K	8.31	1203	166
8	7.10	10K	9.91	1009	142
10	6.90	10K	11.54	867	126
10	7.30	10K	14.51	689	94
15	7.10	5K	11.12	450	63
15	7.05	3K	7.13	421	60

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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.

Date

Signature of Adviser