The *in vitro* Effect of Two Oral Hypoglycemic Agents on Hepatic Protein Synthesis.* (31247)

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A considerable amount of work is reported in the literature on the mechanism of action of the oral hypoglycemic agents in reducing blood glucose levels(1). However, the condition of diabetes mellitus involves disturbances in lipid and protein metabolism as well as the carbohydrate imbalance. Relatively little work has been done on the effect of the oral hypoglycemic agents on protein metabolism. Recant and Fischer(2) reported that oral administration of tolbutamide to rats increased the ability of liver slices to incorporate glycine-C^{14} into protein. On the other hand, Manchester and Young(3) reported that the *in vitro* addition of tolbutamide to rat diaphragm caused a decrease in the incorporation of alanine-C^{14} into the protein of the diaphragm. A confirmation of this finding was reported by Jarrett and Butterfield(4) who also reported that *in vitro* addition of tolbutamide decreased the production of $C^{14}O_2$ from leucine-C^{14} over control values. Inhibition of protein synthesis was recently found to occur in liver homogenates with either tolbutamide or phenethylbiguanide added *in vitro*(5,6). It is noteworthy that *in vitro* addition of insulin has been found to increase the incorporation of radioactive amino acids into rat liver slices(7), but that no direct effect of insulin on a homogenate system has yet been observed(8).

The present study was undertaken to investigate some of the parameters of the inhibition of protein synthesis, and to correlate this with the affect of the oral hypoglycemic agents on leucine catabolism.

Materials and methods. Female albino rats of the Sprague-Dawley strain, fed *ad libitum* on standard laboratory chow, were used. After decapitation, the livers were homogenized in 3 volumes of 0.08 M potassium phosphate buffer, pH 7.4, and centrifuged at 0°C for 10 minutes at 700 × *g* to remove nuclei and cell debris. The supernatant fluid (0.50 ml) was incubated at 37°C for 2 hours in a stoppered 25 ml Erlenmeyer flask containing a center well with 0.50 ml of a solution of the following composition, hereinafter referred to as standard incubation solution:

KCl, 0.075 M; $MgCl_2$, 0.01 M; $KHCO_3$, 0.015 M; glucose, 0.015 M; nicotinamide, 0.02 M; NAD, 2.5×10^{-3} M; ATP, 2×10^{-3} M; final pH 7.4.

A specified amount of leucine-C^{14} (U.L.) was added to each incubation vessel. The center well of each flask contained 0.2 ml of 10% KOH to trap any CO_2 liberated in the course of the incubation. This would serve as a measure of the relative amount of degradation of the isotope, since an early step in the catabolism of leucine involves a decarboxylation reaction.

At the end of each incubation, the reaction was terminated by addition of 5% trichloroacetic acid. The KOH was quantitatively flushed from the center well into 5.0 ml of 10% $BaCl_2$. The resulting precipitate of $BaCO_3$ was filtered using a Tracerlab precipitation apparatus, washed 3 times with distilled water, 3 times with ethanol:acetone (1:1 v/v) and 3 times with acetone. The dried precipitates were weighed and counted in a thin window Geiger-Mueller counter. All samples were corrected for self-absorption.

The precipitated protein was washed once with 5% trichloroacetic acid, 3 times with 95% ethanol, and 3 times with acetone. The final washed precipitate was dissolved in 2.0 ml of 0.1 N NaOH. In every case, 1.0 ml of this solution was taken to dryness under an infra-red lamp and counted in a thin window Geiger-Mueller counter to a 5% probable

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TABLE I. Effect of Presence of Tolbutamide and Phenethylbiguanide on Incorporation of Leucine-C^{14} into Hepatic Protein.

Sample	cpm/mg	Range
Control	237	230–243
"	241	237–245
Tolbutamide (4×10^{-3} M)	118	102–138
Idem	136	124–144
PEBG (4×10^{-3} M)	231	222–239
Idem	214	210–227

Each figure represents the mean of 3 flasks.

error. Aliquots of the solution were analyzed for protein content by the biuret method as given by Gornall *et al*(9). Since the amount of NaOH present was constant for each sample, no corrections were made for self-absorption.

Results. The incorporation of leucine-C^{14} into rat liver protein is inhibited by the presence of 4×10^{-3} *M* tolbutamide, but is essentially unaffected by the presence of 4×10^{-3} *M* phenethylbiguanide (Table I). An attempt was made to determine whether this effect was to be found in both the microsomal bound protein and the soluble protein, or if it was specific for one type. Accordingly, the next incubation was stopped by addition of 5 ml of 5% casein hydrolysate. It was then centrifuged for 1 hour at 100,000 rpm in a Spinco Ultracentrifuge to sediment the particulate proteins. The supernatant was decanted and an aliquot precipitated for protein, washed and counted as usual. The remaining pellet was resuspended in buffer, and an aliquot of this was treated in a similar manner. The results (Table II) indicate that tolbutamide in a concentration of 4×10^{-3} *M* inhibits the incorporation of leucine-C^{14} into both the soluble and particulate protein of rat liver. The same amount of phenethylbiguanide causes no significant difference in either fraction. The relative amounts of incorporation from one fraction to the other are not meaningful, since different aliquots were used.

TABLE II. Distribution of Radioactivity.

Fraction	cpm/mg	Range
Soluble protein		
Control	86	82– 96
"	104	97– 108
Tolbutamide	60	59– 62
"	61	60– 61
PEBG	96	92– 99
"	96	94– 100
Particulate protein		
Control	958	932– 971
"	964	950– 975
Tolbutamide	595	547– 621
"	659	627– 693
PEBG	1039	1004–1072
"	1098	1068–1123

Each figure represents the mean of 3 flasks.

To elucidate these differences further, it was decided to determine the effect of increasing concentrations of the oral hypoglycemic agents on incorporation. Hence, a series of experiments was performed in which 0.5 ml of homogenate was incubated with 0.5 ml of standard incubation solution, 2 μcuries of leucine-C^{14}, and a varying amount of hypoglycemic agent. In addition to measurement of incorporation into protein, the total CO_2 liberated was collected and counted as a measure of the catabolism of leucine. The results (Fig. 1 and 2), show that both tolbutamide and phenethylbiguanide inhibit CO_2 release from leucine as well as protein synthesis. However, the mode of action of the two agents is very different. Whereas tolbutamide causes a linear decrease in CO_2 release, the inhibition induced by phenethylbiguanide is exponential in character. There is a very significant initial decrease in radioactivity which reaches a plateau when approximately 8 μ-moles of PEBG have been added. No such plateau is evidenced with addition of large quantities of tolbutamide, but the initial effect is not nearly so great.

The effects observed on protein incorporation are even more interesting. When small amounts of PEBG are added to the incubation mixture, little or no effect is observed. This is in agreement with the data given in Tables I and II. However, when the solution approaches 8×10^{-3} *M* in PEBG, a very sharp decrease in incorporation occurs, which levels off at a relatively low value. The inhibition of protein incorporation which is induced by tolbutamide appears to be linear with respect to concentration of the agent added.

The possible effect of glucose on the system was then investigated by incubating, in the

usual manner, both in the presence and absence of exogenous glucose (.0075 *M*). The concentration of the oral hypoglycemic agent was 4 $\times$ 10^{-3} *M* in all cases. Results are presented in Tables III and IV.

The data indicate that there is no significant effect of glucose on either protein incorporation of leucine or of CO_2 release from leucine. This is true both in the presence and absence of tolbutamide and phenethylbiguanide.

Discussion. The results indicate that the presence *in vitro* of either tolbutamide or phenethylbiguanide depresses the protein metabolism of rat liver homogenate. An inhibition of both the anabolism and catabolism of leucine-C^{14} is noted. Results further indicate that the method of inhibition is different for the two compounds. Inhibition by tolbutamide of both CO_2 production and protein incorporation is linear with respect to concentration of chemical added. Little more can be said of the tolbutamide inhibition than that it appears to be independent of the presence of exogenous glucose in the medium.

The case of inhibition by phenethylbiguanide is more interesting. This compound exerts a marked inhibitory effect on CO_2 production upon the addition of a small amount which reaches a plateau after further addition of the chemical. Small amounts do not affect the incorporation of leucine-C^{14} into hepatic protein, but once 8 μmoles have been added, there is a sudden and significant decrease in incorporation. It is possible that the two phe-

TABLE III. Effect of Glucose and Oral Hypoglycemic Agents on $C^{14}O_2$ Release.

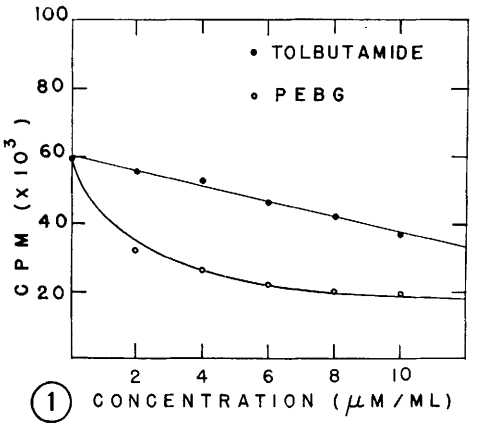
Incubation	cpm/mg
Control	12,592
"	12,751
Control + glucose	12,159
" "	12,938
Tolbutamide	10,429
"	10,540
Tolbutamide + glucose	9,523
" "	9,638
PEBG	4,566
"	4,979
PEBG + glucose	4,737
" "	4,833

Each figure represents the mean of 2 flasks.

TABLE IV. Effect of Glucose and Oral Hypoglycemic Agents on Incorporation of Leucine-C^{14} into Protein.

Incubation	cpm/mg
Control	327
"	333
Control + glucose	352
" "	392
Tolbutamide	231
"	228
Tolbutamide + glucose	249
" "	201
PEBG	274
"	317
PEBG + glucose	254
" "	322

Each figure represents the mean of 2 flasks.



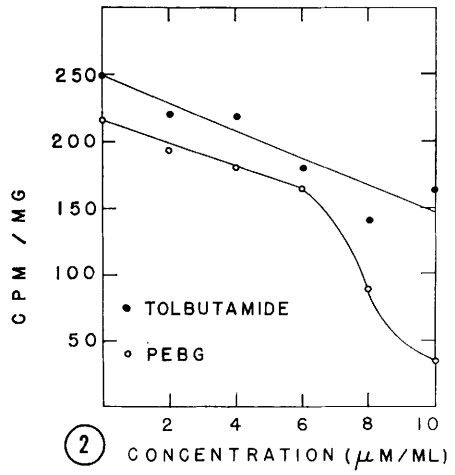


FIG. 1. Effect of concentration of oral hypoglycemic agents on $C^{14}O_2$ release from leucine-C^{14} by rat liver homogenate.

FIG. 2. Effect of concentration of oral hypoglycemic agents on incorporation of leucine-C^{14} into protein by rat liver homogenate.

nomena are related. Perhaps the large initial inhibition of leucine catabolism compensates for whatever anabolic inhibition is occurring. Since less leucine is being degraded, more is available for incorporation and this effect counteracts the process of inhibition of protein synthesis. Once the catabolic inhibition reaches a plateau, the compensation no longer exists, and the inhibition of protein anabolism can assert itself. This hypothesis represents one possibility.

Another explanation is offered for the peculiar instance of PEBG-induced inhibition of protein incorporation. It is possible that there is present in the medium an unknown substance which reacts with the PEBG to form an inactive complex; inactive toward inhibition of protein synthesis, it would be active toward inhibition of CO_2 release. Once 8 μmoles of PEBG were added, all of the unknown substance would be complexed and further addition of PEBG would result in the sudden drop in incorporation seen in Fig. 2. It was originally thought that this unknown substance might be glucose, since there are 8 μmoles of glucose present in each incubation flask. The data in Table IV show the fallacy of this assumption.

Summary. Leucine-C^{14} was utilized to study the affects of 2 oral hypoglycemic agents (tolbutamide and phenethylbiguanide) on protein metabolism. Both compounds were seen to inhibit $C^{14}O_2$ production and incorporation of the amino acid into protein by rat liver homogenate. Differences in the mode of inhibition are noted and discussed. Neither compound appears to be dependent on the presence of glucose for its *in vitro* affects on protein metabolism.

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On Insulin Immunologic Activity in Livers and Kidneys of Mice Injected with Beef Insulin.* (31248)

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In mammals studied to date the I^{131} of intravenously administered Insulin-I^{131} has been found to concentrate rapidly in liver and especially in kidney, and to pass somewhat less rapidly from an original TCA insoluble state to a TCA soluble state(1,2,3,4,5). In the case of liver, the highest concentration of TCA insoluble I^{131} was shown to be in the mitochondrial and microsomal fractions(4). The decreased TCA insolubility of the I^{131} was accompanied by a loss of insulin biological (hypoglycemia-inducing) activity(2). These findings have been interpreted by some (3) to indicate that intact insulin molecules are capable of penetrating rapidly into and being concentrated by liver and kidney cells. A number of arguments have been advanced by Krahl(6), Kallee(7) and others that this interpretation has not been proved. One argument is that the TCA insoluble I^{131} found in liver and kidney after Insulin-I^{131} administration may not represent intact insulin mole-

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