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THE INHIBITION *IN VITRO* OF HEPATIC PROTEIN BIOSYNTHESIS BY VARIOUS ORAL HYPOGLYCEMIC AGENTS—SITE OF ACTION*

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Abstract—The incorporation of leucine-^{14}C into hepatic protein *in vitro* by a microsomal system is inhibited by the presence of tolbutamide, chlorpropamide or phenethylbiguanide in the range of 2–10 mM. This inhibition is also evidenced if lysine-^{14}C or phenylalanine-^{14}C is substituted for the leucine-^{14}C tracer. Studies on the incorporation of isotope into both soluble and microsomally bound protein indicate that the inhibition is not concerned with the final step in which nascent protein is released from the ribosome. Other experiments involving the preincubation of labelled amino acid with the cell sap indicate that the inhibition occurs after the formation of the amino acyl-*s*-RNA complex. It is concluded that both the arylsulfonylureas and the guanidine derivatives inhibit protein synthesis at the microsomal level.

IN THE PAST decade or so, a number of oral hypoglycemic compounds have gained prominence as a substitute for insulin in the treatment of certain milder types of diabetes mellitus. Two of the most interesting of these agents, representing different classes of chemical compounds, are tolbutamide and phenethylbiguanide (PEBG). Whereas the former is thought to exert its effect by stimulating the deficient pancreas to produce more insulin, the latter almost certainly acts by a different mechanism.[1] Nonetheless, both of these compounds are able adequately to control the blood sugar level of mild diabetics when administered orally.

The condition of diabetes mellitus is characterized not only by a defect in carbohydrate metabolism, but also by malfunctions in lipid and protein metabolism. Although there has been a great deal of work devoted to the influence of insulin on protein biosynthesis,[2, 3] the problem of the influence of the oral hypoglycemic agents on protein biosynthesis has been largely ignored. In 1957, Recant and Fischer[4] reported an experiment involving the oral administration of tolbutamide to rats for a period of 5 days. On the final day, the animals were sacrificed and the incorporation of glycine-^{14}C into liver slice protein was measured and compared with that of normal rats. The authors reported that the ability of the liver slices from the tolbutamide-treated animals to incorporate amino acids into protein was significantly greater than that of liver slices derived from normal animals. They further noted that a single acute injection of tolbutamide produced no effect. Recent experiments by DeChatelet

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and McDonald[5] have questioned these findings by demonstrating that the injection of either tolbutamide or PEBG could result in an inhibition of hepatic protein synthesis. Under no circumstances was a stimulation of protein synthesis observed.

Manchester and Young noted in 1958[6] that the incorporation of alanine-^{14}C into isolated rat diaphragm was depressed in the presence of either tolbutamide or carbutamide regardless of whether glucose or pyruvate was present in the medium. A concentration of tolbutamide of 10 mM gave an incorporation which was only 9 per cent of the control level.

In 1964, Jarrett and Butterfield[7] amplified the experiments of Manchester and Young. They reported that a concentration of tolbutamide of 10^{-2} M depressed the incorporation of leucine-^{14}C into the protein of diaphragm from normal rats. This effect was marginal at a tolbutamide concentration of 10^{-3} M and was not seen at lower concentrations.

These results were extended to a liver homogenate system by DeChatelet and McDonald[8] who reported that both tolbutamide and PEBG depressed the incorporation of leucine-^{14}C into hepatic protein independently of the glucose concentration. More recent experiments[9] have demonstrated that this inhibition of leucine incorporation is also evident in a microsomal system derived from the livers of either normal or alloxan-diabetic rats.

This report is concerned with the mechanism of action of these agents in inhibiting the incorporation of leucine-^{14}C into hepatic protein.

MATERIALS AND METHODS

Female albino rats of the Holtzman strain, fed *ad libitum*, were sacrificed by decapitation and the livers were quickly removed and immersed in ice-cold buffer. Microsomes were isolated by the procedure described by Campbell and Kernot.[10] Incubations were performed at 37° in an Elmac incubator-shaker apparatus for a sufficient time to ensure maximum incorporation of isotope into protein (Fig. 1). Each incubation

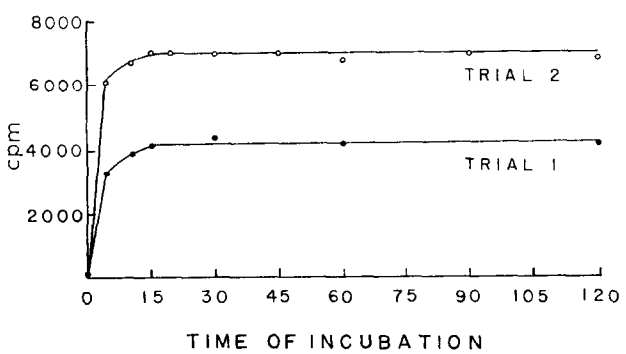

FIG. 1. Effect of time of incubation, in minutes, upon the incorporation of leucine-^{14}C into hepatic protein. (Each point represents the mean of 2 determinations.)

flask contained the following in a total volume of 1·0 ml: 0·40 ml microsome suspension (representing the microsomes from 1 g liver); 0·30 ml cell sap (total protein approximately 17 mg/flask); 7·0 μmoles Mg^{2+}; 2·0 μmoles ATP; 0·5 μmoles GTP; 30·0 μmoles creatine phosphate; 50 μg creatine phosphokinase; and 1·0 μc

leucine-^{14}C (u.l., L-isomer, sp. act. 230 μc/μmole). These concentrations were experimentally determined to give optimal incorporation of the isotope into protein. The inclusion of a mixture of 19 unlabelled amino acids gave no enhancement of incorporation, indicating a sufficiency of these compounds in the cell sap. Tolbutamide and chlorpropamide were added as the sodium salt; PEBG was added as the hydrochloride. The reaction was initiated by the addition of microsomes to flasks containing the cofactors, cell sap and hypoglycemic agents.

The reactions were terminated by the addition of 1·0 ml of 10% trichloroacetic acid and the resulting precipitate was washed as previously described.[9] The final washed precipitate was dissolved in 2·0 ml of 0·1 N sodium hydroxide and a 1·0-ml aliquot was taken to dryness and counted in a thin window Geiger–Mueller counter to a 5 per cent probable error.

RESULTS

The incorporation of L-leucine-^{14}C into hepatic protein is depressed by the addition of tolbutamide, chlorpropamide or PEBG, as indicated by the data in Table 1. The

TABLE 1. EFFECTS OF THE ADDITION *IN VITRO* OF THREE ORAL HYPOGLYCEMIC AGENTS ON THE INCORPORATION OF LEUCINE-^{14}C INTO HEPATIC PROTEIN*

Concn (mM)	Average cpm incorporated in the presence of		
	Tolbutamide	Chlorpropamide	PEBG
0	4412	4412	4412
2	4000	3796	3586
4	3425	3526	3281
6	2939	2761	3039
8	2376	2392	2739
10	1895	1820	2331
15		1073	1690

* Each point represents the average of 2 determinations.

question arises as to whether this is a generalized effect on protein biosynthesis or whether it is a phenomenon peculiar to leucine-^{14}C. For example, the compounds added might increase leucine catabolism without having any direct effect on protein biosynthesis. In order to rule out such possibilities, the effects of tolbutamide and PEBG on the incorporation of three different labelled amino acids into protein were studied. The data in Table 2 show that these agents inhibit the incorporation of leucine-^{14}C, lysine-^{14}C and phenylalanine-^{14}C into hepatic protein. This strongly suggests that the effect of these agents is on protein biosynthesis rather than on some aspect of amino acid metabolism.

Although the previous experiments demonstrated that certain concentrations (2–10 mM) of the oral hypoglycemic agents inhibit protein synthesis, this did not exclude the possibility that other lower concentrations might have exactly the opposite effect.

Duncan and Clark[1] reported that the blood level of arylsulfonylurea required to elicit a hypoglycemic response *in vivo* equals 10–15 mg per 100 ml (0·31 to 0·51 mM). This would imply that the concentrations used in the previous experiments were considerably above the "physiological" blood concentration. Similarly, they report

that the blood level of PEBG required for a hypoglycemic response is only 5 μg/ml (0·021 mM). This would seem to imply that a totally unrealistic amount of PEBG was used in the previous experiments, except that Wick *et al.*[11] have reported that this compound is greatly concentrated in the liver upon injection, so that we would expect a hepatic concentration of PEBG to be considerably above the circulating blood concentration.

TABLE 2. EFFECT OF THE ADDITION *IN VITRO* OF TOLBUTAMIDE AND PHENETHYLBIGUANIDE ON THE INCORPORATION OF LEUCINE-^{14}C, LYSINE-^{14}C AND PHENYLALANINE-^{14}C INTO THE HEPATIC PROTEIN OF NORMAL RATS*

Incubation conditions	Leucine-^{14}C (cpm)	Lysine (cpm)	Phenylalanine (cpm)
Control	3420	1336	2648
Control	3371	1252	2329
Tolbutamide (10 mM)	1452	615	1741
Tolbutamide (10 mM)	1367	604	1838
PEBG (10 mM)	1918	852	1503
PEBG (10 mM)	1900	669	1572

* Each amino acid had the same specific activity (50 μc/μmole), 1·25 μ was added to each flask.

Experiments run with these lower concentrations of hypoglycemic agents demonstrated no appreciable effect on the incorporation of leucine-^{14}C into hepatic protein. The concentration of tolbutamide and chlorpropamide in these experiments was 0·4 mM; the concentration of PEBG was 0·02 mM. A slight inhibition, of the order of 10 per cent, was seen in the case of chlorpropamide and PEBG, but the significance of this is doubtful. At any rate, it is certain that this lower concentration of the agents does not stimulate protein synthesis as might be desired for these antidiabetic compounds.

The next experiment was designed to test whether the oral hypoglycemic agents inhibited incorporation of leucine-^{14}C into both soluble and microsomally bound protein or into only one of these fractions. Incubations were performed as usual in the presence and absence of oral hypoglycemic agents. At the end of the incubation period, the reactions were stopped by the addition of 2·0 ml of cold 1% L-leucine followed by chilling of the flasks in an ice bath. The contents of each flask were transferred quantitatively to an ultracentrifuge tube. All tubes were centrifuged at 40,000 rpm for 1 hr to sediment the microsomes. At the end of this time, the clear supernatant was decanted and the pellet was rinsed once with 1·0 ml of cold buffer. This rinse was added to the supernatant and the total was taken to represent soluble protein. Similarly, the microsomal pellets were resuspended in cold buffer and protein was precipitated in all tubes by the addition of trichloroacetic acid to a final concentration of 5 per cent. The resulting precipitates were washed and counted in the usual way.

Table 3 shows the results obtained with both tolbutamide and PEBG at two different concentrations. The first thing to be noted is that most of the newly synthesized protein is still microsomally bound and relatively few counts have been released into the medium, in spite of the fact that the time of incubation was 2 hr. It is apparent, however, that each of these agents inhibits biosynthesis of both the

soluble and the microsomal protein. Further, the inhibition seen in the soluble protein is of approximately the same order of magnitude as that seen in the microsomal protein (for 20 mM tolbutamide, soluble protein is 24 per cent of control value while microsomal protein is 22 per cent of control; for 20 mM PEBG, soluble protein is 29 per cent of control value while microsomal protein is 19 per cent of control).

TABLE 3. EFFECT OF THE ADDITION *IN VITRO* OF TOLBUTAMIDE AND PHENETHYLBIGUANIDE ON THE INCORPORATION OF LEUCINE-^{14}C INTO SOLUBLE AND MICROSOMALLY-BOUND PROTEIN*

Incubation conditions	Average cpm incorporated into	
	Soluble protein	Microsomal protein
Control	228	1611
Tolbutamide (6 mM)	170	1308
PEBG (6 mM)	172	1550
Control	579	8903
Tolbutamide (20 mM)	138	1961
PEBG (20 mM)	172	1725

* Each point represents the mean of 3 determinations.

These data eliminate the release step of the protein from the ribosome as a possible site of action for either of these agents. If they blocked the release of the finished protein, there would be a large inhibition seen in the soluble protein and little or none in the microsomal fraction. Since this was not observed, we can conclude that neither agent acts by inhibiting the release of the completed protein from the ribosome.

The final series of experiments was designed in an attempt to localize further the site of action of the oral hypoglycemic agents. The procedure for these experiments differed considerably from that previously described. In each experiment, the cell sap was preincubated with the isotope and cofactors for a period of 15 min. The experimental variables fell into three groups. One series of flasks contained only cell sap and isotope; after 15 min of preincubation the reaction (protein synthesis) was begun by the addition of microsomes. These flasks served as controls. A second series contained cell sap, isotope and 6 μmoles of oral hypoglycemic agent during the preincubation; the reaction was initiated by the addition of microsomes. The third series contained only cell sap and isotope during the preincubation; the reaction was initiated by the simultaneous addition of 6 μmole of oral hypoglycemic agent and microsomes.

The rationale of the procedure is as follows: during the preincubation, the reactions which occur in the cell sap alone can take place, i.e. the steps in the biosynthetic pathway leading to the formation of the soluble RNA–amino acid complex. If a compound inhibits one of these steps, its presence during the preincubation should result in an inhibition; but if it is added with the microsomes, no inhibition would occur because the initial reactions have already taken place. That this is reasonable is demonstrated by the data in Fig. 1, which indicate that the incorporation of isotope into protein is virtually complete in 15 min. Hence we are justified in assuming that the *s*-RNA–amino acid complex would be formed during this time interval.

The data in Tables 4–6 indicate the results of these experiments employing three different oral hypoglycemic agents: tolbutamide, chlorpropamide or PEBG. In all three cases, the incorporation of leucine-^{14}C into hepatic protein is depressed by the same amount, regardless of whether the agent is present during the preincubation or is added along with the microsomes. This leads to the conclusion that all three agents

TABLE 4. SITE OF ACTION *IN VITRO* OF THE TOLBUTAMIDE INHIBITION OF HEPATIC PROTEIN SYNTHESIS

Incubation conditions	Experiment 1 (cpm)	Experiment 2 (cpm)
Control: no tolbutamide added	895	1453
	1004	1391
	825	1422
Tolbutamide present during preincubation	710	879
	694	901
	645	932
Tolbutamide added with microsomes after preincubation	610	915
	646	917
	611	892

TABLE 5. SITE OF ACTION *IN VITRO* OF THE CHLORPROPAMIDE INHIBITION OF HEPATIC PROTEIN SYNTHESIS

Incubation conditions	cpm
Control: no chlorpropamide added	4043
	4280
	3846
Chlorpropamide present during preincubation	2683
	2698
	2727
Chlorpropamide added with microsomes after preincubation	2415
	2534
	2457

TABLE 6. SITE OF ACTION *IN VITRO* OF THE PHENETHYLBIGUANIDE INHIBITION OF HEPATIC PROTEIN SYNTHESIS

Incubation conditions	Experiment 1 (cpm)	Experiment 1 (cpm)
Control: no PEBG added	1429	1351
	2252	1462
	1298	1412
PEBG present during preincubation	1101	1192
	1094	1098
	1024	1102
PEBG added with microsomes after preincubation	909	981
	873	1078
	1049	1069

exert their inhibition somewhere at the microsomal level and excludes the possibility that they affect either the activation of the amino acids or the transfer of the amino acid to *s*-RNA.

One final observation is noteworthy regarding these experiments. The absolute level of incorporation in all cases (including controls) is lower than that usually achieved in other experiments. It was postulated that this was due to the preincubation period itself, i.e. some heat-labile factor was being destroyed during this period before the microsomes were added, resulting in an overall decrease in protein synthesis. To test this possibility, microsomes and cell sap were isolated from the livers of normal rats and were preincubated together at 37°. At varying periods of time, aliquots were withdrawn and used to start the actual incubation by addition to flasks containing the cofactors and isotope. The incubation was then allowed to proceed for 1 hr before being terminated by the addition of 10% trichloroacetic acid.

The results in Fig. 2 indicate that there is, indeed, a heat-labile factor present in the mixture which is adversely affected by the 37° temperature of the incubation bath.

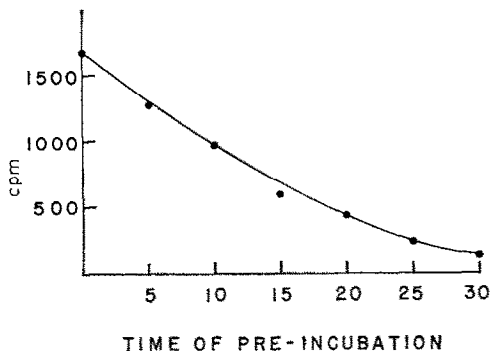

FIG. 2. Effect of preincubation time, in minutes, upon the incorporation of leucine-^{14}C into hepatic protein. (Each point represents the mean of 3 determinations.)

This might explain the lower values of incorporation encountered in the experiments which involved a preincubation step.

DISCUSSION

The arylsulfonylureas reduce the fasting blood sugar level of intact and partially pancreatectomized or mildly alloxan-diabetic animals, but they do not do so after total pancreatectomy in dogs, rabbits, rats or man, or in animals made severely diabetic with alloxan.[12, 13] These observations have been interpreted as indicating that tolbutamide (and allied arylsulfonylureas) acts by stimulating the pancreas to produce more insulin. The mode of action of PEBG differs markedly from that of the arylsulfonylureas as evidenced by the fact that this compound is an effective hypoglycemic agent in pancreatectomized animals,[14] in severe alloxan diabetes[15] and in patients with juvenile or insulin-deficient types of diabetes.[16] Evidently, PEBG does not require that functional pancreatic tissue be present. This fact excludes the possibility that it acts by stimulating the beta cells to produce more insulin, as has been proposed for tolbutamide.

It has been observed, however, that each of these compounds, when added *in vitro*, has a similar inhibitory effect on the incorporation of leucine-^{14}C into hepatic protein when used with the microsomes and cell sap isolated from normal rat liver. A measurement of the effects of the compounds on both soluble and microsomally bound protein showed that incorporation was depressed equally in both fractions, indicating that

both the tolbutamide- and PEBG-induced inhibition occurred before the protein was released from the ribosome, i.e. the compounds did not affect the release step in the biosynthetic pathway.

Other experiments in which the leucine-^{14}C was charged by preincubation with the cell sap indicated that the inhibition in both cases occurred after the formation of the amino acyl–*s*-RNA complex. In other words, both tolbutamide and PEBG, when added *in vitro*, inhibit the incorporation of leucine-^{14}C into hepatic protein somewhere at the level of the microsomes. This is not to say that they both act in precisely the same manner, for this is a very complex sequence of reactions. For example, one compound might cause disaggregation of the ribosomes while the other might affect the attraction between the amino acyl–*s*-RNA and the template. Nonetheless, both agents are seen to act at approximately the same point in inhibiting the biosynthesis *in vitro* of hepatic protein.

These similarities are unexpected in view of the known different mechanisms of action of the two agents with respect to carbohydrate metabolism. This only serves to emphasize the fact that the effect of the oral hypoglycemic agents on protein metabolism is not a secondary phenomenon dependent upon the effect on carbohydrates.

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