Effects of Tolbutamide, Mesoxalate and Phenformin in Vitro on the Liberation of Nitrogen by Rat Liver Slices

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The in vitro effects of tolbutamide on the liver slice and diaphragm of the rat have been extensively studied. The majority of reports record various measurements of the metabolism of glucose. In both isolated tissue and intact animals there is evidence[1,2] that tolbutamide decreases hepatic glucose production, and that this is due in part to a reduced rate of glycogenolysis.[1,3] Since gluconeogenesis from protein is an important component of hepatic glucose production, we have attempted to measure the direct action of tolbutamide on the production of nonprotein nitrogen in vitro by rat liver slices. Similar observations have been made on the rat diaphragm, and the effects of mesoxalate[4,5] and phenformin have been examined by way of comparison.

Kaufmann and Wertheimer[6] have reviewed earlier studies on the liberation of nitrogen by liver slices and have reported the influence of different media and of several physiological conditions on the protein and nonprotein nitrogen output. Their experience will be recalled in connection with our methods and results.

METHODS

Liver slices and hemidiaphragms prepared from 125 gm. male Wistar rats starved eighteen to twenty-four hours were incubated in Krebs-Ringer bicarbonate solution, which contained 200 mg. per cent U-C^{14}-glucose. Kaufmann and Wertheimer[6] found that "liver slices incubated in Krebs-Ringer bicarbonate medium release 48 per cent more total nitrogen than those incubated in Krebs-Ringer phosphate medium," and they describe the effects of other alterations in medium. The presence of 200 mg. per cent of glucose did not change the release of total nitrogen in the phosphate medium which they used. The use of Krebs-Ringer bicarbonate thus appears to be suitable when comparative results are sought, although absolute figures will differ with different media. The effect of tolbutamide or urea was tested by adding sodium tolbutamide in a final concentration of 20 mg. per cent, or urea in a final concentration of 150 mg. per cent. Addition of tolbutamide or urea caused no change in the *p*H of the medium. The rats were killed by cervical fracture. The liver was removed and washed in cold Krebs-Ringer bicarbonate solution which contained no glucose. The left lobe of the liver was sliced with a Stadie-Riggs slicer and the slices were washed in cold Krebs-Ringer bicarbonate solution. The slices were blotted gently on filter paper, weighed, and placed in a Warburg vessel containing 3.0 ml. medium with isotopic glucose. The liver slices weighed about 200 mg. The usual time between sacrifice and incubation was five minutes. Filter papers (20 x 40 mm.) were placed in the center wells of the Warburg vessels which were gassed with a mixture of 95 per cent oxygen: 5 per cent CO_2 for five minutes, and stoppered. The Warburg vessels also contained ground-glass side-arms which were stoppered by rubber self-sealing penicillin stoppers in order that acid and alkali could be introduced into the vessel at the end of the experiment to permit collection of CO_2 as described below. The vessels were shaken for three hours at 37° C. and 80 cycles per minute in a Dubnoff metabolic incubator. After three hours incubation, 0.3 ml. of 9N NaOH was placed in the center well through the side-arm containing the rubber stopper by means of a needle and syringe. Then 0.5 ml. of 4N H_2SO_4 was added directly to the medium with another syringe and the vessels were shaken again at 37° C. for twenty minutes to permit absorption of CO_2 by the filter paper.

Kaufmann and Wertheimer[6] employed Kline's[7] method of deducting the value of an initial incubation period, a procedure designed to account for physical diffusion and leakage due to injury of the tissue. In using the beginning of incubation as the zero value instead of a fifteen-minute[7] or thirty-minute[6] point, allowance has not been made for the large initial output of nitrogen

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due to handling the tissue. This will be discussed in connection with the results.

Determination of the conversion of C^{14}-glucose to $C^{14}O_2$. The filter paper was removed quickly from the center well and placed in a graduated, stoppered centrifuge tube which contained 2 ml. of distilled water. One milliliter of distilled water was used to rinse out the center well and added to the centrifuge tube. The final volume was made to 3.0 ml. A 0.5 ml. aliquot of this was plated, dried and counted.

Determination of incorporation of C^{14}-glucose into glycogen. The liver slice was removed from the vessel, placed in a test tube which contained 1.0 ml. of 30 per cent KOH, and heated in a water bath for one hour. Then two drops of 10 per cent $ZnSO_4$ and 2.5 ml. of ethanol were added, warmed and placed in an ice bath for thirty minutes. The glycogen was precipitated by centrifugation, dissolved in water and reprecipitated by ethanol twice more. The precipitate was then dissolved in 2 ml. of 1N H_2SO_4 and hydrolyzed in a water bath for two hours at 100° C. The hydrolysate was neutralized with 30 per cent KOH and the final volume was made up to 3.0 ml. A half milliliter of this hydrolysate was plated, dried and counted. The other 2.0 ml. was used for glucose determination by the Harding modification of the Schaffer-Hartmann method.

Determination of NPN *in medium.* The medium was transferred to a 15 ml. centrifuge tube by Pasteur pipette and centrifuged. A 2.0 ml. aliquot of the supernatant was added to 2 ml. of 10 per cent sodium tungstate, shaken vigorously, and centrifuged. In the experiments with urea, 2.0 ml. of medium and 2.0 ml. of 10 per cent sodium tungstate were added to a 10-ml. graduated cylinder and the final volume was made to 10.0 ml. with distilled water. After filtration the nitrogen was determined by a micro-Kjeldahl method. Urea was determined by the method of Reinhold and Gentzkow.[8] This measures urea plus ammonia formation by the isolated tissue, but the results are referred to as urea N. Amino nitrogen was determined by the method of Frame, Russell and Wilhelmi.[9] In each experiment with tolbutamide or urea, the initial nitrogen content of the incubating medium was determined in the same way and the appropriate correction made to determine the amount added by the liver slices. In the studies on tissues from pretreated animals, the amounts of drug are noted in the tables. In in vivo studies, tissues were usually taken one to two hours after the last injection of tolbutamide or mesoxalate. Glutamic oxalic transaminase and glutamic pyruvic transaminase were determined in the Pepper Laboratory of the Hospital of the University of Pennsylvania by the method of Karmen[10] and the results reported in Karmen units.

RESULTS

Liberation of nitrogen. Table 1 shows that tolbutamide reduced the nonprotein nitrogen output by liver slices of normal rats. The decrease amounted to 18 per cent. If this were a metabolic effect in the intact animal, it would account for an appreciable decrease in the daily hepatic glucose production from protein. In the diaphragm there was an even greater output of nitrogen, which was less affected by tolbutamide. Sodium mesoxalate exerted no such action on the liver (table 2).

The interpretation of this measurement of nitrogen output requires more knowledge of its metabolic meaning. Kline[7] discussed this and showed that much nitrogen entered the medium during the first fifteen minutes of incubation, and that this initial loss "is not of biological significance." To eliminate this nonmetabolic nitrogen, he used the fifteen-minute period of incubation as the zero point. In addition to the time element, the forms of nitrogen have received consideration. Kline[7] determined total nitrogen. Kaufmann and Wertheimer,[6] using a thirty-minute fore-period, examined the release of protein and of nonprotein nitrogen. Under their experimental conditions (fasting, alloxan diabetes, etc.), the release of nonprotein nitrogen was constant, and protein nitrogen was the variable component. To relate the effects of tolbutamide to these observations,[6,7] total nitrogen and nonprotein nitrogen of the medium were determined at thirty minutes and at three hours and thirty minutes, as shown in table 3. For both the half-hour and three-hour periods the amount of total nitrogen exceeds that of nonprotein nitrogen, confirming the fact that loss of protein is the major source of the nitrogen liberated by these isolated tissues. By estimating the rate per half hour during the final three-hour incubation, one finds that both forms of nitrogen are liberated more than twice as rapidly during the first half-hour, in agreement with others.[6,7] Tolbutamide had no certain effect in the first half-hour and its effect in the subsequent three-hour period was obscured when measured by total nitrogen. However, the definite effect on nonprotein nitrogen output was again observed, and amounted to a decrease of 17 per cent, essentially the same as in table 1.

To pursue the mechanism of this action of tolbutamide, the output of amino nitrogen and urea was determined. In this work the effect of tolbutamide was compared with that of insulin and of phenformin. The

TABLE 1

Effects of tolbutamide on nonprotein nitrogen (NPN) formation and on the conversion of C^{14}-glucose to CO_2 and glycogen in liver and diaphragm of rats

NPN output Mg. per 100 mg. liver per 3 hr.		Glycogen Mg. per 100 mg.		Glucose C^{14} to CO_2		Glucose C^{14} to glycogen	
				μM per 100 mg. liver per 3 hr.			
Control	Tolbutamide	Control	Tolbutamide	Control	Tolbutamide	Control	Tolbutamide
A. Liver slices in vitro*							
.226†±.007 (16)‡	**.185**§±.008 (16)	.057±.010 (16)	.029±.006 (16)	1.52±.12 (16)	1.76±.14 (16)	1.68±.27 (16)	**0.70**±.11 (16)
B. Liver slices of rats pretreated with tolbutamide‖							
		.057±.010 (16)	.040±.008 (9)	1.52±.12 (16)	2.67±.37 (9)	1.68±.27 (16)	1.35±.29 (9)
C. Hemidiaphragms in vitro*							
.336±.007 (11)	.309±.008 (11)	.166±.010 (11)	.128±.007 (11)	5.16±.62 (11)	6.20±.57 (11)	7.22±.71 (11)	6.00±.50 (11)
D. Hemidiaphragms of rats pretreated with tolbutamide‖							
		.166±.010 (11)	.163±.014 (9)	5.16±.62 (11)	4.16±.43 (9)	7.22±.71 (11)	7.35±.36 (9)

*Tolbutamide, 20 mg. per cent concentration in medium.
†Mean and standard error.
‡Number in parentheses is number of animals used.
§Bold type indicates significant difference from control (P=<0.01).
‖Tolbutamide, 10 mg. per 100 gm. rat once daily for three days before experiment.

TABLE 2

Effects of sodium mesoxalate on rat liver slices and hemidiaphragms

NPN output Mg. per 100 mg. liver per 3 hr.		Glycogen Mg. per 100 mg.		Glucose C^{14} to CO_2		Glucose C^{14} to glycogen	
				μM per 100 mg. liver per 3 hr.			
Control	Mesoxalate	Control	Mesoxalate	Control	Mesoxalate	Control	Mesoxalate
A. Liver slices in vitro*							
.223†±.012 (11)‡	.228±.010 (11)	.043±.009 (11)	.040±.009 (11)	1.57±.24 (11)	1.82±.28 (11)	1.66±.26 (11)	1.82±.25 (11)
B. Liver slices of rats pretreated with mesoxalate§							
		.043±.009 (11)	.026±.003 (8)	1.57±.24 (11)	**3.86**‖±.61 (8)	1.66±.26 (11)	.80±.15 (8)
C. Hemidiaphragms in vitro*							
		.206±.018 (6)	.196±.011 (6)	4.67±.40 (6)	4.56±.60 (6)	5.23±.55 (6)	5.33±.40 (6)
D. Hemidiaphragms of rats pretreated with mesoxalate§							
		.206±.018 (6)	.220±.014 (8)	4.67±.40 (6)	4.35±.45 (8)	5.23±.55 (6)	6.98±.71 (8)

*Sodium mesoxalate, 1.0 mg. per cent concentration in medium.
†Mean and standard error.
‡Number in parentheses is number of animals used.
§Sodium mesoxalate, 0.5 mg./100 gm. rat per day for three days before experiment.
‖Bold type indicates significant difference from control (P=<0.01).

results provide a clear differentiation between the effects of tolbutamide and phenformin on the liver as tested here. The lack of any immediate effect of insulin on the liver slice is expected.[11] Tolbutamide reduces the amino acid output of the liver slice by an amount which accounts for approximately a quarter of the reduction in NPN output. Tolbutamide had no action on urea formation. In contrast, phenformin had no influence on the liberation of amino nitrogen, but strikingly reduced urea production. (Parenthetically, if the control

TABLE 3
Effect of tolbutamide on normal rat liver slices

Initial thirty minutes Mg. per 100 mg. liver per thirty minutes				Subsequent 3-hr. incubation Mg. per 100 mg. liver per 3 hr.			
Total N		NPN		Total N		NPN	
Control	Tolbutamide	Control	Tolbutamide	Control	Tolbutamide	Control	Tolbutamide
.178*±.017	.164±.011	.069±.008	.061±.003	.432±.095	.413±.020	.194±.003	**.161**†±.004

*Mean and standard error; six animals used. Tolbutamide, 20 mg. per cent concentration in medium.
†Bold type indicates significant difference from control (P=<0.01).

figures for urea production are expressed in micromoles, they agree with those of Stadie's[12] report.) When the livers of rats pretreated with phenformin were tested (last line, table 4), a significant depression in urea formation was also seen. This might represent the situation in the treated patient.

Since tolbutamide is a sulfonylurea and since urea as the end product of deamination might inhibit the reaction, the influence of an excess of urea in the medium was tested. In a concentration of 150 mg. per cent, urea caused no change in the nonprotein nitrogen output of liver slices. In two normal cats the fasting excretion of urinary nitrogen was measured, with and without three daily doses of 100 mg. of tolbutamide, but no difference was demonstrated. Even with this large dosage (at eight-hour intervals) the possible adjustment of protein metabolism between doses was recognized. Nitrogen balance has not been influenced in man by tolbutamide.[13]

Since a reduction of amino nitrogen output by the liver slice might be the result of inhibition of transaminases, the effect of tolbutamide on the liberation into the medium of two transaminases was determined. Table 5 presents the results which indicate that tolbutamide suppresses the ability of the liver to produce or liberate these enzymes. Control studies showed that tolbutamide in the concentrations employed in these determinations does not alter the activity of the enzymes themselves, and thus the results are due to an action on the tissue. Four determinations of the transaminase activity of homogenates of liver slices after incubation with or without tolbutamide failed to reveal any difference. These preliminary observations with homogenates differ from the inhibitory effect of tolbutamide found on alanine:α-ketoglutaric acid transamination, reported by Bornstein.[14] However, the results with the simple nonprotein nitrogenous substances studied and those on transaminase in media all support

TABLE 4
Effects of tolbutamide, insulin and phenformin on the output of three nitrogenous products by liver slices

Amino N—mg. per 100 mg. of liver slice per three hours		NPN—mg. per 100 mg. of liver slice per three hours		Urea N—mg. per 100 mg. of liver slice per three hours	
Control	Tolbutamide*	Control	Tolbutamide	Control	Tolbutamide
.043†±.002 (15)‡	**.035**§±.001 (15)	.180±.006 (15)	**.149**±.004 (15)	.037±.002 (22)	.035±.001 (22)
Control	Insulin‖	Control	Insulin	Control	Insulin
.045±.002 (12)	.039±.002 (12)	.188±.007 (12)	.164±.007 (12)	.037±.002 (13)	.035±.002 (13)
Control	Phenformin**	Control	Phenformin	Control	Phenformin
.039±.002 (9)	.048±.002 (9)	.188±.005 (9)	**.059**±.003 (9)	.040±.004 (6)	**.006**±.001 (6)
.042±.002 (36)	.048††±.002 (6)	.185±.004 (36)	.180±.006 (6)	.038±.002 (19)	**.023**±.001 (6)

*Tolbutamide 20 mg. per cent concentration in medium.
†Mean and standard error.
‡Figures in parentheses show number of rats used.
§Bold type indicates significant difference from control (P=<0.01).
‖0.1 unit per ml. in medium.
**Phenformin 0.5 mg. per ml. medium.
††In this series the rats were pretreated with phenformin (see text).

TABLE 5

Effect of tolbutamide on transaminase output of liver slices*

Glutamic oxalacetic transaminase			Glutamic pyruvic transaminase		
Units per 100 mg. liver slice per three hours					
Control	Tolbutamide	Tolbutamide effect	Control	Tolbutamide	Tolbutamide effect
20.9±1.6	16.9±1.6	—3.9±0.5†	4.2±0.5	3.3±0.4	—0.9±0.3‡

*Six rats used in each series. Tolbutamide, 20 mg. per cent concentration in medium. Mean and standard error, except tolbutamide effect which is difference between means and the standard error of the difference. Transaminase determinations performed by the William Pepper Laboratory, Hospital of the University of Pennsylvania.
†P=<.01.
‡P=<.05.

the concept that these hypoglycemic drugs act in a significant degree by the inhibition of gluconeogenesis.

In short, tolbutamide does not immediately alter the liberation of protein or urea but nevertheless causes an appreciable reduction of amino nitrogen and of other unknown components of nonprotein nitrogen. Phenformin does not act on amino nitrogen but inhibits urea formation (deamination).

Changes in CO_2 production and glycogen content. Table 1 shows that the glycogen content of both tissues was slightly reduced by tolbutamide and that the incorporation of glucose-C^{14} from the medium into glycogen was reduced in three of the four experimental conditions (table 1: A, B, C). The conversion of labeled glucose to CO_2 was slightly increased. Due to differences in methods, the results are not comparable to those of Ashmore et al.,[1] who found an increase in liver and muscle glycogen in fasted and fed normal rats one hour after pretreatment with tolbutamide. The increased utilization of carbohydrate by the liver, as measured by CO_2 production, agrees with the report of Recant and Fisher,[15] who inferred an increased utilization from the increased hepatic vein pyruvate.

Mesoxalate (table 2) did not alter glycogen content. In the liver, glucose utilization was increased as estimated by labeled CO_2 production, but the incorporation of glucose-C^{14} into glycogen followed no clear pattern.

COMMENT

Tolbutamide added in vitro decreases the output of nonprotein nitrogen by liver slices of normal rats. There is no change in the loss of protein or in the output of urea, but a reduction in amino nitrogen output accounts for about a quarter of this effect. Neither urea nor mesoxalate, added to the medium in the concentrations tested, have such an effect. The smaller but possibly valid effect on the nonprotein nitrogen output of diaphragm (table 1) suggests that this action is not limited to the liver but may apply to other tissues to a greater or lesser extent. If a reduction of nonprotein nitrogen of the order of 18 per cent occurred continually in patients treated with sulfonylureas, it should be a significant part of the metabolic effect. However, if this be so, it is not reflected in the nitrogen balance in our casual trials in the cat or in man.[18] On the other hand, a decreased hepatic nitrogen output is in accord with the increased incorporation of labeled glycine into liver protein reported by Recant and Fisher,[15] and with the diminished output of glucose by the liver after tolbutamide.[1] A level of tolbutamide in vitro, corresponding to that reported here, caused a 20 per cent depression in glucose production by rabbit liver slices.[16] The authors state that this supports the idea that there is an inhibition of glycogenolysis, but in table 1 above, liver glycogen and the incorporation of labeled glucose from the medium into glycogen were lowered, suggesting the primacy of an action on some component of protein metabolism. The ultimate meaning of these various findings requires further study.

Phenformin was found to behave differently. It caused a greater reduction in NPN output, a striking reduction in urea production and had no effect on amino nitrogen. These laboratory yardsticks clearly indicate differences between the effects of tolbutamide and phenformin on the liver slice. In the case of the rats pretreated with phenformin (last line, table 4), 10 mg. per 100 gm. were given twenty-four and two hours before the liver was taken. In these experiments, phenformin was not added in vitro.

In connection with other studies on these drugs, it is clear that, except for a few pretreated animals, the effects reported above took place in the presence of whatever insulin was bound to the tissues but in the absence of added insulin secretion.

SUMMARY

The addition of tolbutamide to the incubating medium caused a reduction in the liberation of nonprotein nitrogen by liver slices and to a lesser degree by dia-

phragms of normal rats. There was no change in the output of protein or urea by liver slices, but that of amino nitrogen was reduced, as was the transaminase activity of the medium. Sodium mesoxalate had no such action on nitrogen output. The simultaneous changes in glycogen content, conversion of labeled glucose in medium to CO_2, and to glycogen, were recorded but could not be clearly related to the changes in nitrogen metabolism. Phenformin caused a greater reduction in the output of nonprotein and urea nitrogen, but had no effect on amino nitrogen. Both tolbutamide and phenformin affect the liver but do so in different ways as measured by nitrogen metabolism in vitro.

SUMMARIO IN INTERLINGUA

Effectos in Vitro de Tolbutamida, Mesoxalato, e Phenformina Super le Liberation de Nitrogeno per Trenchos de Hepate de Ratto

Le addition de tolbutamida al terreno de incubation causava un reduction in le liberation de nitrogeno non ligate a proteina ab trenchos de hepate de rattos normal e, minus pronunciatemente, ab le diaphragma de tal animales. Esseva notate nulle alteration in le rendimento de proteina o de urea per le trenchos de hepate, sed illo de amino-nitrogeno esseva reducite, e un reduction esseva etiam constatate in le activitate transaminasic del terreno. Mesoxalato de natrium non habeva iste effecto in le rendimento de nitrogeno. Le simultanee alterationes del contento de glycogeno e le conversion de marcate glucosa del terreno in CO_2 e glycogeno esseva registrate sed non poteva esser relationate clarmente al alterationes del metabolismo de nitrogeno. Phenformina causava un plus grande reduction in le rendimento de nitrogeno non ligate a proteina e de nitrogeno de urea sed habeva nulle effecto super le nitrogeno de aminas. Tanto tolbutamida como etiam phenformina affice le hepate, sed in tanto que le metabolismo de nitrogeno in vitro permitte mesurar lo, le maniera in que ille effecto occurre es differente in le duo casos.

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