Alloxan Diabetes and Insulin Effects on Amino Acid Incorporating Activity of Rat Liver Microsomes.* (26255)

WILLIAM S. ROBINSON† (Introduced by M. E. Krahl)
Department of Physiology, University of Chicago, Ill.

In alloxan-diabetic rats there is a progressive decrease in peptide-synthesizing capacity of the liver(1,2). The specific question to be tested here was whether any component of the microsomal peptide synthesizing system(3-5) could be shown to be altered in activity by lack or excess of insulin in the liver donor.

Materials and methods. Two types of experiments were done. In one type microsomes were prepared from 300 g male Sprague-Dawley diabetic rats previously given alloxan and maintained on insulin 3 weeks(6); during this period each rat was removed once from insulin and food for 24 hours and its fasting blood sugar determined; those with values above 300 mg% were selected. Seventy-two hours before use diabetic rats were separated into 2 groups: one group was continued on insulin sufficient to prevent glycosuria (control diabetics); the other received no insulin and developed hyperglycemia and glycosuria (untreated diabetics); both were fasted in metabolism cages 24 hours before use. Urinary ketones were estimated semi-quantitatively as acetoacetic acid(7). At sacrifice, blood sugars(8,9) were estimated on tail vein specimens; all untreated diabetics had fasting blood sugars above 300 mg%. In the second type of experiment, normal 300 g rats were treated with excess insulin: 8 units protamine-zinc-insulin (Lilly) and 4 units regular insulin at 20 hours, and again with 2 units regular insulin at 4 hours, before sacrifice; these animals were fasted 24 hours before use.

The microsomes, the supernatant fraction, and the fraction containing amino acid activating enzymes (precipitated at pH 5 from the supernatant fraction and redissolved at pH 7.6) were prepared according to Zamecnik *et al.*(3,4,10,11), with 0.35 M sucrose-0.05 M Tris buffer, pH 7.6, as the homogenizing medium. The microsome fraction from each liver was resuspended in the buffered sucrose; its protein concentration was estimated(12) and adjusted by dilution so that equal amounts of microsome protein could be incubated; aliquots were also analysed for protein nitrogen, phospholipid phosphate and ribonucleic acid(13). A stock pH 5 enzyme preparation from normal liver was used throughout, unless otherwise specified. Microsome fractions were incubated in triplicate 20 minutes at 37°C as shown in Tables I and IV, after which ice-cold 10% trichloroacetic acid was added. Protein was then isolated and its radioactivity estimated(14). The concentration above which there was no further increase in incorporation was ascertained for each component of the medium, and each was subsequently used at a concentration above this to insure that only the activity of the microsomes would be rate-limiting.

Results. Control experiments indicated that incorporation of leucine-1-C^{14} into microsomal protein, as here measured, was an enzyme catalysed process: (a) no incorporation occurred up to one hour at 2°C, nor at zero time at 37°C; (b) incorporation increased linearly with time up to one hour at 37°C; (c) ATP (adenosinetriphosphate), GTP (guanosine triphosphate), an ATP-generating system (*e.g.*, phosphoenol pyruvate and pyruvate kinase), and a fraction of soluble liver protein were required; (d) incorporating activity of microsomes and activating activity of the soluble fraction were lost by heating either fraction at 55°C for 5 minutes; (e) no incorporation occurred when pH 5 enzyme fraction was incubated without microsomes.

* This investigation was aided by grants from Life Insurance Medical Research Fund, Eli Lilly and Co., and Dr. Wallace C. and Clara A. Abbott Memorial Fund, Univ. of Chicago.

† Present address: Dept. of Medicine, Columbia University College of Physicians and Surgeons, New York.

Diabetic animals. In each of 2 experiments microsomes from livers of untreated diabetic rats were less active in amino acid incorporation than those from control (insulin treated) diabetics (Table I). In both series loss of amino acid incorporating activity in microsomes was associated with development of ketonuria in the untreated diabetic rats (Table I). No difference was found with respect to ability of soluble fractions to activate microsomal incorporation (Table II).

In microsomes from untreated diabetic rats there was a decrease in ratio of phospholipid phosphate to protein nitrogen, and a slight increase in ratio of RNA to protein nitrogen relative to control diabetics (Table III). This suggests that in the diabetic animal the synthesis of endoplasmic reticulum lipoprotein may be depressed.

TABLE I. Effects of Insulin Withdrawal from Alloxan-Diabetic Rats on Amino Acid Incorporating Activity of Liver Microsomes: Group 1, Maintained on Insulin (See Text); Group 2, Insulin Withdrawn for 72 Hr. Each incubation sample in Exp. 1 contained 5 mg microsomal protein, 1.5 mg pH 5 enzyme protein from normal rat liver, 0.02 mg pyruvate kinase protein, 10 μmoles phosphoenol pyruvate, 1.0 μmole ATP, 0.25 μmole GTP, 5 μmoles Mg^{++} ion, and 0.9 μmole DL-leucine-1-C^{14} with 4.5 × 10^6 cpm in 1 ml incubation medium. Conditions were the same in Exp. 2, except that 6 mg microsomal protein was used.

Group	Rat No.	Blood sugar, mg %	Urinary ketones, mg %	Mean specific radioactivity of microsomal protein, cpm/mg
		Exp. 1		
1	1	Not	0	381
	2	measured	0	346
	3		0	321
	4		0	314
	5		0	307
	6		0	276
			Mean	324 ± 15*
2	1	404	10	326
	2	364	10	312
	3	364	30	257
	4	360	30	234
	5	380	30	187
	6	384	80	172
			Mean	248 ± 26*
		Exp. 2		
1	1	Not	0	303
	2	measured	0	279
	3		0	276
	4		0	273
	5		0	226
			Mean	271 ± 13*
2	1	312	10	212
	2	316	80	190
	3	376	80	167
	4	368	300	145
	5	640	300	113
			Mean	165 ± 17*

* Stand. error. Difference between Groups 1 and 2 is significant to $P < 0.02$ in Exp. 1 and $P < 0.001$ in Exp. 2.

TABLE II. Comparison of Activating Effects of Pooled Soluble Fractions from 6 Normal Rats and 6 Untreated Diabetic Rats (Group 2, Exp. 1, Table I). Each incubation sample contained: 6.0 mg normal microsomal protein, 3.0 mg pooled soluble liver protein, 20 μmole creatine phosphate, 1.0 μmole ATP, 0.25 μmole GTP, 5.0 μmole Mg^{++}, and 0.5 μmole DL-leucine in 1 ml.

Normal microsome donor No.	Mean specific radioactivity of microsomal protein, cpm/mg: With soluble fraction from Normals	From Diabetics
1	134	150
2	144	156
3	154	172
4	186	175
5	185	185
6	206	186
Mean	167 ± 11*	171 ± 6

* Stand. error. Difference between means is not significant.

Excess insulin. Insulin treatment of normal rats prior to sacrifice resulted in increased amino acid incorporating activity of the liver microsomal fraction (Table IV). Absolute values in Table IV cannot be compared with those of Table I because the whole soluble supernatant fraction was used as activator in Table IV, and the pH 5 fraction in Table I.

No effects of insulin incubated in the medium with normal liver microsomes could be demonstrated, the mean values for 4 experiments without and with 0.01 unit insulin (Lilly crystalline, lot 657501) per ml being 136 ± 13 and 132 ± 7 cpm/mg microsomal protein, respectively. The incubation conditions were the same as for the normal series of Table II except that 6.5 mg normal liver microsomal protein were used in each vessel.

Discussion. Impaired amino acid incorporation into microsomes from diabetics, and the increased incorporation into microsomes from insulin-treated normal animals, appears to represent some change of enzyme activity of the microsomal fraction. These variations

TABLE III. Composition of Microsomes from Alloxan-Diabetic Rats: Group 1, Maintained on Insulin (See Text); Group 2, Insulin Withdrawn for 72 Hr. Diabetic animals are the same as those of Table I, Exp. 2. Values for normal animals are those of Palade and Siekevitz(13).

Group	mg RNA / mg protein N	μg phospholipid P / mg protein N
1	.84 ± .058*	152 ± 10*
2	1.09 ± .045*	110 ± 6*
Normal rats	1.12	157
	.91	152
	.99	133

* Mean ± stand. error, 5 rats/group. Differences between Groups 1 and 2 are significant to $P < 0.007$ for each ratio.

do not stem from variations in size of a free leucine pool, as the microsome pellets were well washed; neither are they the consequence of differences in activity of soluble protein or pH 5 fraction, as these were prepared from normal liver and equal amounts were added to all incubation samples in a given experiment.

The correlation of impairment of amino acid incorporating activity with increasing ketosis in the liver donor suggests that capacity of the liver to metabolize fat normally and ability to synthesize protein fail concurrently. Whether or not there is a causal relation between these 2 defects remains to be investigated.

TABLE IV. Effect of Insulin Treatment of Normal Rats on Amino Acid Incorporating Activity of Liver Microsomes: Group 1, Normal Controls; Group 2, Insulin Treated (See Text). Each incubation sample contained 5 mg microsomal protein, 4 mg soluble protein from normal liver, 1 μmole ATP, 0.025 μmole GTP, 5 μmoles Mg^{++} ion, and 0.9 μmole DL-leucine-1-C^{14} with 4.5×10^6 cpm in 1 ml medium.

Group	Rat No.	Mean specific radioactivity of microsomal protein, cpm/mg
1	1	115
	2	98
	3	96
	4	88
	Mean	99 ± 6*
2	1	181
	2	156
	3	148
	4	133
	Mean	155 ± 10*

* Mean ± stand. error. Difference between Groups 1 and 2 is significant to $P < 0.002$.

After the present study was initiated, Korner(15) demonstrated a decreased ability of liver microsomes from hypophysectomized rats to incorporate labeled amino acids into protein. Normal incorporating activity was restored by administration of insulin prior to removal of the tissue. Doell(16) reported a 40 to 50% increase in amino acid incorporating activity of microsomes after injection of insulin into normal non-fasting rats.

Summary. (1) Liver microsomes from severely diabetic rats incorporate leucine-1-C^{14} into protein at a lower rate than microsomes from diabetic control rats maintained on insulin. The former have also a lower phospholipid-P and a higher RNA content per unit of protein-N. (2) Microsomes from normal rats incorporate amino acids at a higher rate after injection of insulin. (3) These changes occurred specifically in microsomes; no difference was found with respect to ability of soluble fractions to activate microsomal incorporation. Insulin was without effect upon the microsomal system *in vitro*.

ADDENDUM: Korner(17) found amino acid incorporating capacity of liver microsomes from a different type of diabetic rat to be defective.

1. Krahl, M. E., *J. Biol. Chem.*, 1953, v200, 99.
2. ———, *Recent Progress Hormone Research*, 1956, v12, 199.
3. Zamecnik, P. C., Keller, E. B., *J. Biol. Chem.*, 1954, v209, 337.
4. Littlefield, J. W., Keller, E. B., Zamecnik, P. C., *ibid.*, 1955, v217, 111.
5. Campbell, P. N., Greengard, O., Kernot, B. A., *Biochem. J.*, 1960, v74, 107.
6. Spiro, R. G., Hastings, A. B., *J. Biol. Chem.*, 1958, v230, 751.
7. Free, H. H., Free, H. M., *Am. J. Clin. Path.*, 1958, v30, 7.
8. Somogyi, M., *J. Biol. Chem.*, 1952, v195, 19.
9. Nelson, N., *ibid.*, 1944, v153, 375.
10. Hoagland, M. B., Keller, E. B., Zamecnik, P. C., *ibid.*, 1956, v218, 345.
11. Keller, E. B., Zamecnik, P. C., *ibid.*, 1956, v221, 45.
12. Gornall, A. G., Bardawill, G. J., David, M. M., *ibid.*, 1949, v177, 751.
13. Palade, G. E., Siekevitz, P., *J. Biophys. Biochem. Cytol.*, 1958, v2, 171.

14. Wool, I. G., Krahl, M. E., *Am. J. Physiol.*, 1959, v196, 961.
15. Korner, A., *Biochem. J.*, 1960, v74, 471.
16. Doell, R., *Fed. Proc.*, 1959, v18, 37.
17. Korner, A., *J. Endocrinology*, 1960, v20, 256.

Received September 16, 1960. P.S.E.B.M., 1961, v106.

A Non-Hydrazine Monoamine Oxidase Inhibitor with Antihypertensive Properties. (26256)

DAVID HORWITZ AND ALBERT SJOERDSMA
Section on Experimental Therapeutics, National Heart Institute, Bethesda, Md.

Previous studies in this laboratory have suggested that effective inhibition of monoamine oxidase (MAO) by various drugs in human hypertensives results in an orthostatic decrease in blood pressure(1-3). Agents of this type include iproniazid (Marsilid), trans - DL - 2-phenylcyclopropylamine (SKF-385, Parnate), β-phenylisopropylhydrazine (JB-516, Catron), phenylzine (Nardil), nialamide (Niamid), DL-serine-N^2-isopropylhydrazide (RO-4-1038)(4), and isocarboxazid (Marplan)(5). However, any final conclusion concerning a relationship between enzyme inhibition and blood pressure lowering has been tempered by the fact that, with the exception of SKF-385, all of these agents are hydrazine derivatives. Also, either because of low potency or occurrence of prohibitive toxicity, none have become valuable therapeutic agents in hypertension.

Thus, the discovery of the potent, non-hydrazine, MAO inhibitor, N-benzyl-N-methyl-2-propynylamine·HCl (MO-911), by Taylor *et al.*(6) was of special interest. This report presents initial results of administering this compound to human subjects. The findings give added support to the hypothesis that pronounced MAO inhibition produces an orthostatic decrease in blood pressure in hypertensive patients. Barring unforeseen toxicity, MO-911 appears to be a promising antihypertensive agent.

Materials and methods. The subjects were 4 females and 5 males, aged 26 to 51 years, with sustained hypertension uncomplicated except for renal insufficiency (pt. C.F.), polycystic kidneys (pt. C.M.) and unilateral pyelonephritis (pt. W.C.). Except for occasional one- to 3-day passes, the subjects were hospitalized throughout the periods of study. MO-911 was supplied as 25 mg scored tablets.* The only other medications were cardiac glycosides, barbiturates for sleep and salicylates. Salt intake was moderately restricted in one patient and calories in another.

Blood pressures (arm cuff technic) and pulse rates were measured 4 times daily in the supine position and also after 2 minutes of quiet standing. Control observations were made over a period of at least 6 days. Then, MO-911 was administered orally as a single dose before breakfast each day for periods of 14 to 34 days. Initial dosage was 75 to 125 mg/day with a downward revision when pronounced decline of blood pressure developed. With one exception, observations were extended for 8 to 17 days after cessation of therapy; during this time blood pressure levels approached or had returned to pre-treatment values.

Serial blood counts, urinalyses, tests of renal and hepatic function and periodic screening of visual acuity and color vision were performed to detect possible toxic effects. Degree of MAO inhibition was evaluated by daily assays of urinary tryptamine and tyramine(3).

Results. It was quickly established that MO-911 is an effective antihypertensive agent as well as a potent MAO inhibitor. Its long duration of action permitted achievement of sustained effects with use of single daily doses.

Effects on blood pressure and pulse rate. A pronounced fall in standing blood pressure

* Through the courtesy of Dr. R. K. Richards, Abbott Laboratories, N. Chicago, Ill.