4. Homburger, F., Tregier, A., *Endocrinology,* 1957, v61, 627.

5. Villee, C. A., *Ann. N. Y. Acad. Sci.,* 1959, v75, 524.

6. Homburger, F., Tregier, A., Grossman, M. S., *Endocrinology,* 1957, v61, 634.

7. Snell, G., *J. Nat. Cancer Inst.,* 1953, v13, 151.

Received June 29, 1959. P.S.E.B.M., 1959, v102.

Comparison of Tolbutamide and Insulin Pre-Treatment Prior to Alloxan Induced Diabetes. (25267)

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Introduction of sulfonylurea compounds as oral hypoglycemic agents in therapy of mild diabetes has stimulated numerous studies to determine the mechanism of action of these drugs. Comprehensive symposia of these works have been published(1-4). Many investigators interpreted their findings as demonstrating that these drugs stimulate release of insulin by the pancreas and produce their hypoglycemic effect by increasing amount of available insulin. Insulin and glucose partially protect starved animals from alloxan diabetes(5) while starvation potentiates diabetogenic activity(6). This study was undertaken to compare the effect of insulin pre-treatment with tolbutamide pre-treatment on the diabetogenic potency of alloxan in rats.

Methods. Sprague-Dawley virgin female rats, weighing between 130-180 g, were used. In any one series the average weight of individual groups within that series did not vary by more than 5 g. The diabetogenic agent (alloxan-monohydrate) was injected subcutaneously on the basis of its anhydrous weight at a dose of either 150 or 175 mg/kg of body weight. Glucose was given either orally *ad lib* or injected intraperitoneally at a dose of 5 g/kg of body weight in the form of a 20% aqueous solution. Tolbutamide* was injected subcutaneously in all groups at a dose level of 200 mg/kg at a site on the opposite side of the body from that of the alloxan injection. Commercial crystalline zinc insulin was diluted in saline and injected subcutaneously at a dose of 1 unit/kg. A modified Somogyi-Nelson technic(7,8) was used for blood sugar determinations. Blood was taken from the tail of non-fasting animals from 24 hr through 6 days after administration of alloxan. Normal blood sugar range was found to be between 60 and 120 mg%, using the above technic. Microscopic sections were prepared of the pancreas, kidney and liver from animals in each group studied. All tissues were stained with hematoxylin-eosin (Harris). In addition the pancreatic tissues were stained according to Gomori for beta granules. For liver glycogen studies the animals were sacrificed by breaking the neck. Duplicate liver samples were rapidly removed, weighed and homogenized in 30% KOH. Glycogen determinations were done by the method of Hawk, Oser and Summerson(9). *Series I.* Following fasting period of 48 hr 15 animals were divided into 3 equal groups and additionally pre-treated as follows: 1) no further treatment; 2) glucose oral *ad lib* 6 hr; 3) tolbutamide 200 mg/kg subcutaneously followed by glucose oral *ad lib* 6 hr. The above pre-treatment was followed by subcutaneous injection of 175 mg/kg of alloxan. *Series II.* After 48 hr fasting period another series of animals were divided into 4 groups and further pre-treated as follows: 1) no additional treatment; 2) glucose oral *ad lib* 3 hr; 3) tolbutamide 200 mg/kg followed by glucose oral *ad lib* 3 hr; 4) insulin 1 unit/kg followed by glucose oral *ad lib* 3 hr. Alloxan at a dose level of 150 mg/kg was injected subcutaneously into all 4 groups following the above pre-treatment. *Series III.* Thirty animals were fasted 48 hr before intraperitoneal injection of 5 g/kg of glucose. These rats were then divided into 3 equal groups and treated

* Kindly supplied by Dr. C. J. O'Donovan of the Upjohn Company, Kalamazoo, Mich.

TABLE I. Effect of Various Pre-treatments on the Survival of Alloxan Treated Rats.

Series 1—Alloxan 175 mg/kg

Pre-treatment	Survived Avg blood sugar (mg %) 48 hr post-alloxan	Died Avg blood sugar (mg %) 48 hr post-alloxan	P values† *vs* 1	P values† *vs* 2
Starvation 48 hr	0	5 743 (2)*		
Idem + glucose oral *ad lib* 6 hr pre-alloxan	3 664	2 1200	.04	
Idem and tolbutamide 200 mg/kg 6 hr pre-alloxan	0	5 730 (3)	1.00	.04

Series 2—Alloxan 150 mg/kg

Pre-treatment	Survived Avg blood sugar (mg %) 72 hr post-alloxan	Died Avg blood sugar (mg %) 72 hr post-alloxan	P values† *vs* 1	P values† *vs* 2	P values† *vs* 3
Starvation 48 hr	5 491 (2)*	5 415 (3)			
Idem + glucose oral *ad lib* 3 hr pre-alloxan	5 398 (4)	4 356 (3)	.83		
Idem and insulin 1 u/kg 3 hr pre-alloxan	6 180 (1)	4 396 (4)	.65	.90	
Starvation 48 hr + glucose oral *ad lib* and tolbutamide 200 mg/kg 3 hr pre-alloxan	0	8 337 (5)	.02	.01	.01

Series 3—Alloxan 150 mg/kg

Pre-treatment	Survived Avg blood sugar (mg %) 48 hr post-alloxan	Died Avg blood sugar (mg %) 48 hr post-alloxan	P values† *vs* 1	P values† *vs* 2
Starvation 48 hr + glucose 5 g/kg I.P. 3 hr pre-alloxan	10 459	0		
Idem and insulin 1 u/kg 3 hr pre-alloxan	10 397	0	1.00	
Starvation 48 hr + glucose 5 g/kg I.P. and tolbutamide 200 mg/kg 3 hr pre-alloxan	3 466	7 752	.005	.005

* No. of animals on which blood sugars were obtained; all other averages represent total number. † Chi square probability.

as follows: 1) no further treatment; 2) insulin, 1 unit/kg; 3) tolbutamide, 200 mg/kg 3 hr after the above pre-treatment, 150 mg/kg of alloxan was injected subcutaneously. *Series IV.* Thirty animals were fasted 48 hr, divided into 3 equal groups and further treated as in Series 3. Two additional rats from each of the above groups, as well as 2 starved and 2 non-treated animals were used for liver glycogen determinations.

Results. The results in Series 1 (Table I) indicate that addition of tolbutamide altered the protective effect of glucose against the lethal action of alloxan. The difference between these groups was found to be significant ($p = 0.04$). In Series 2 (Table I) mortality rate of glucose-tolbutamide group was significantly greater than any of the other 3 groups ($p = 0.02$). Results in Series 3 (Table I) show no difference between glucose and glucose plus insulin treated groups. The group receiving glucose plus tolbutamide had an increased mortality rate significantly higher than either the glucose or insulin treated group, ($p = 0.005$).

In Table I blood glucose values are shown for each of the 3 series of experiments. All animals studied developed moderate to severe diabetes regardless of the treatment. All animals which died had blood sugar levels as high, if not higher, than those which survived. It was also observed that rats having levels in excess of 700 mg% rarely lived. Microscopic sections of the pancreas showed complete degranulation in all animals which died. These findings appear to indicate that death

TABLE II. Summation of Comparable Groups in Series 1, 2 and 3.

Pre-treatment	No. of animals	Survived	Died	P values* vs total	vs 1	vs 2	vs 3
Starvation 48 hr	15	5	10	.25			
Idem + glucose oral *ad lib* or I.P.	29	20	9	.005	.02		
Idem and insulin	20	16	4	.005	.005	.45	
Starvation 48 hr + glucose oral *ad lib* or I.P. and tolbutamide	28	3	25	.005	.07	.005	.005
Total	92	44	48				

* Chi square probability.

was a result of diabetes.

A summation of comparable groups in Table I is shown in Table II. In the fasted and the tolbutamide pre-treated groups most of the animals died. Conversely in the glucose and the insulin pre-treated groups over 70% of the animals lived. The fasted and/or the tolbutamide group differed significantly from either the glucose or insulin treated group.

Series 4 was treated the same as Series 3 but failed to show severe diabetes and no deaths occurred. There was no apparent explanation for the difference in response between the 2 series. A comparison of blood sugar levels 5 days following alloxan injection is shown in Table III. The frequency of hyperglycemia is not significantly different between the glucose and the glucose plus insulin pre-treated groups. The increased incidence of hyperglycemia in the tolbutamide pre-treated group is most likely different from the insulin group ($p = 0.075$).

The liver glycogen studies show that the animals receiving I.P. glucose following starvation attain a normal level of liver glycogen. The starved animals had approximately 10% of the normal value. The insulin or tolbutamide pre-treatment resulted in liver glycogen approximately 60% of the non-treated group.

Discussion. We found a consistently greater mortality or hyperglycemia in starved or glucose plus tolbutamide pre-treated animals than in glucose or glucose plus insulin pre-treated groups. Regardless of route of glucose administration these differences remained unaltered. Because the insulin and tolbutamide pre-treated animals had similar liver glycogen content it may be assumed that the differences in alloxan response are not related to liver glycogen. If tolbutamide stimulates and insulin suppresses pancreatic activity, it might appear that the extent of activity at time of alloxan injection would explain the differences. However, glucose which stimulates pancreatic release of insulin has an alloxan inhibitory effect similar to insulin and opposite from tolbutamide. Starvation and tolbutamide both potentiate alloxan. Assuming pancreatic stimulation by tolbutamide and no stimulation by starvation, the secretory activity again cannot be correlated with the diabetogenic activity of alloxan. Regardless of the mechanism, when animals are pre-

TABLE III. Effect of Various Pre-treatments on Blood Sugar and Liver Glycogen Levels in Alloxan Treated Rats. Series IV.

Pre-treatment	Alloxan, mg/kg	Liver glycogen, %	Blood sugar (mg %) 5 days post-alloxan: Above 120	Below 120	P values* vs 3	vs 4
Normal (non-treated)		2.39				
Starvation 48 hr		.22				
Idem + glucose 5 g/kg I.P. 3 hr pre-alloxan	150	2.32	4	6		
Idem and insulin 1 u/kg 3 hr pre-alloxan	150	1.46	3	7	.65	
Starvation 48 hr + glucose 5 g/kg I.P. and tolbutamide 200 mg/kg 3 hr pre-alloxan	150	1.54	7	3	.20	.07

* Chi square probability.

treated prior to alloxan induced diabetes, tolbutamide has an effect different from insulin.

The present findings may be due to a toxic effect and not related to pancreatic function; however daily doses in rats as high as 500 mg/kg of tolbutamide for 9 months have shown no histological changes in the internal organs except the pancreas(10).

Summary. 1) Starvation or glucose plus tolbutamide potentiates the diabetogenic activity of alloxan with increased mortality. 2) Glucose alone or glucose plus insulin protects against alloxan diabetes. 3) Different results obtained with insulin or tolbutamide cannot be explained on the basis of liver glycogen content, blood glucose, or theoretical status of pancreatic activity at time of alloxan injection.

1. Peck, F. B., *Diabetes,* 1957, v6, 1.
2. Levine, R., *Ann. N. Y. Acad. Sci.,* 1957, v71, 3.
3. Levine, R., Duncan, G. G., *Metabolism,* 1956, v5, pt. 2, 721.
4. Best, C. H., *Canad. M.A.J.,* 1956, v74, 975.
5. Arteta, J. L., Konig, C., Carballido, A., *J. Endocrinol.,* 1954, v10, 342.
6. Kass, E. H., Waisbren, B. A., PROC. SOC. EXP. BIOL. AND MED., 1945, v60, 303.
7. Somogyi, M., *J. Biol. Chem.,* 1945, v160, 62.
8. Nelson, N., *ibid.,* 1944, v153, 375.
9. Hawk, P. B., Oser, B. L., Summerson, W. H., *Practical Physiological Chemistry,* McGraw-Hill Book Co., N. Y., 13th ed., 1954.
10. Bander, A., *Ann. N. Y. Acad. Sci.,* 1957, v71, 152.

Received June 29, 1959. P.S.E.B.M., 1959, v102.

Transfer of Acquired Immunological Tolerance of Skin Homografts in Mice Joined in Parabiosis.* (25268)

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Acquired immunological tolerance has been produced in several species of animals(1-4). As originally defined, the phenomenon is produced by exposing animals to antigens during period of immunological immaturity. Immunological tolerance to subsequent tissue or organ homotransplantation has usually been achieved by injection of the prospective recipient with viable hematopoietic cells from the prospective donor. In most instances spleen cells have been used, injected either subcutaneously or intraperitoneally during embryonic life or intravenously in the immediate neonatal period. Hasek's technic(4) of embryonal parabiosis has also been effective in production of immunological tolerance. Tissues successfully homotransplanted following establishment of immunological tolerance have been skin in mice(1,2,5), skin in fowl(6), skin in rats(3,7), skin in dogs(8), ovaries(9), adrenals(10) and hypophyseal glands in mice (11). Moreover, mice have been made tolerant to homologous adenocarcinoma by subcutaneous injection of tumor cells in the neonatal period or by injection of viable homologous spleen cells from the prospective donor strain prior to birth, followed later by graft of the tumor from the donor strain (12). To date, then, all examples of true immunological tolerance have been achieved by exposure to donor strain antigen prior to delivery or in the immediate neonatal period. Recently, however, we observed that weanling Z(C3H) mice, injected with viable spleen cells taken from (Z x Ce) F_1 hybrid mice, became tolerant and accepted skin grafts from (Z x Ce) F_1 hybrid donors(13). Similarly, female mice of the A and C57 Bl (subline 1) strains were made tolerant of male skin isografts, either by intravenous injection of male spleen cells in older animals or by establishing a parabiotic union of adult females with isologous males(14). Finally, using the technic of parabiotic union, it has also been demonstrated that adult mice growing homolo-

* Aided by grants from Minnesota Division of Am. Cancer Soc., U.S.P.H.S., and the Am. Cancer Society.