TABLE I.
Anticoagulant Activity of Trypsin Inhibitor from Soya Bean Flour.

Reagents							
Crystalline inhibitor (mg)	Whole blood (cc)	Citrated plasma (cc)	$CaCl_2$ (0.25%) (cc)	Fibrinogen (cc)	Thromboplastin (cc)	Thrombin (cc)	Clotting time
*0	2						8 min.
0.2	2						18 "
†0		0.1	0.15				6 "
†0.3		0.1	0.15				21 "
0		0.1	0.15		0.1		13 sec.
0.1		0.1	0.15		0.1		20 "
†0				0.1		0.1	10 "
†0.5				0.1		0.1	10 "

* 0.2 cc of saline added.
† Total volume of 1 cc in test tube, by addition of imidazole buffer at pH 7.4.

brought to pH 7.4 by NaOH N/10. The results obtained with the crude material were confirmed with a purified preparation 4 times recrystallized,[2] secured through the courtesy of Dr. Moses Kunitz.

All experiments were conducted at pH 7.2-7.4 on human blood, citrated plasma and fibrinogen, in imidazole buffer, at 37°C. The streptolysin was a dry powder, dissolved in saline.*[5] Control experiments showed that the anticlotting and antiproteolytic activity were not due to the small amount of salt present in the crystalline preparation and that the addition of an excess of calcium did not modify the anticlotting activity. Autoclaving of the crude material at 120°C for 20 minutes completely abolished the anticoagulant activity and almost completely the antiproteolytic activity.

These facts represent a new instance of the inhibition of the clotting of blood by trypsin inhibitors.[6,7]

* Supplied by the Commission on Acute Respiratory Diseases, through the courtesy of M. H. Kaplan.

[6] Grob, D., *J. Gen. Physiol.*, 1943, **26**, 423.

[7] Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 373.

15347

Protective Effect of Glutathione and Cysteine Against Alloxan Diabetes in the Rat.*

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Although Wiener[1] reported that alloxan produces fatal convulsions in rabbits, the mechanism of death was not understood until Jacobs[2] demonstrated that injections of alloxan into rabbits resulted in fatal hypoglycemia. It only became apparent that alloxan could produce experimental diabetes when Dunn, Sheehan, and McLetchie[3] reported that alloxan produces necrosis of the

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[1] Wiener, H., *Arch. f. exp. Path. u. Pharm.*, 1899, **42**, 375.

[2] Jacobs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 404.

[3] Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B., *Lancet*, 1943, **1**, 484.

beta cells of the islets of Langerhans. Many workers have since confirmed these observations.

The mechanism of alloxan action is not understood. Since alloxan combines with sulfhydryl groups of protein[4] and since many enzymes require sulfhydryl groups for their activity,[5,6,7] it seemed possible that necrosis might result from enzyme inactivation by alloxan. Thus, it is noted that alloxan inhibits the activity of succinic dehydrogenase[5] as well as the proteolytic enzymes, papain and cathepsin.[6] Active sulfhydryl groups are also necessary for pyruvate oxidation and condensation, malate and ketogluterate oxidation, d-amino acid and l-glutamic acid oxidation, and various fatty acid oxidations.[7]

Because many of the enzymes inactivated by alloxan can be reactivated, in part at least, by adding glutathione,[5,7] a naturally occurring sulfhydryl compound, it was decided to test the effect of glutathione on the course of alloxan diabetes. Cysteine was used for part of the study because it is much cheaper than glutathione and because the sulfhydryl group of glutathione is contained in the cysteine component of the molecule.

In a preliminary note[8] I reported that the injection of alloxan (200 mg/kg) intraperitoneally into rats immediately following an intravenous dose of neutralized cysteine or glutathione (7.5 mM/kg) did not cause diabetes. The injection of alanine, glycine, phosphate buffer, ascorbic acid, or succinic acid in place of the cysteine or glutathione did not protect the animals against alloxan. Leech and Bailey[9] reported that the injection of 300-400 mg/kg of glutathione simultaneously with alloxan did not protect rabbits from diabetes, and Weinglass *et al.*[10] reported that giving 300 mg/kg of alloxan subcutaneously simultaneously with 500 mg/kg of cysteine-HCl did not afford protection to rats. However, it is to be noted that the doses of cysteine and glutathione which I used were much greater than those used by other investigators, for 7.5 mM/kg is equivalent to 912 mg/kg of cysteine and 2,500 mg/kg of glutathione.

Methods. In the preliminary studies alloxan was injected intraperitoneally. The intraperitoneal injection of alloxan into rats in doses of 200 mg/kg was not found to be reliable, for, although this dose usually produced diabetes in about 80% of the rats, occasionally an entire group of animals failed to respond. In contrast, intravenous injections of alloxan in doses of 40 mg/kg produced diabetes in 95% of the animals.[11] Therefore, this dose and route of administration were used in the present studies.

To facilitate the intravenous injection, vasodilation was induced by placing the rats in a 56°C oven for several minutes. A 2% solution of alloxan monohydrate was injected into the tail vein and blood was always drawn into the syringe before the injection was started. Solutions of cysteine hydrochloride or glutathione were carefully neutralized to pH 7.4 with NaOH and diluted so that each cc contained 0.2 to 0.75 mM. Usually one cc of solution was injected per 100 g of body weight. The cysteine was given either before or after the alloxan and different veins were used for each injection. As controls the following compounds were used and were injected in place of cysteine or glutathione:

1. NaCl (7.5 mM/kg), because NaCl is formed in the neutralization of cysteine-HCl.
2. Phosphate buffer, pH 7.4 (2.5 mM/kg), to rule out the effect of pH. Doses larger than 2.5 mM/kg caused a convulsive death, apparently due to hypocalcemic tetany.
3. Alanine, to test whether the protective

[4] Lieben, F., and Edel, E., *Biochem. Z.*, 1933, **259**, 8.

[5] Hopkins, F. G., Morgan, E. S., and Lutwak-Mann, C., *Biochem. J.*, 1938, **32**, 1829.

[6] Purr, A., *Biochem. J.*, 1935, **29**, 13.

[7] Barron, E. S. G., and Singer, T. P., *J. Biol. Chem.*, 1945, **157**, 221; Singer, T. P., and Barron, E. S. G., *J. Biol. Chem.*, 1945, **157**, 241.

[8] Lazarow, A., *Anat. Rec.*, 1945, **91**, 24.

[9] Leech, R. S., and Bailey, C. C., *J. Biol. Chem.*, 1945, **157**, 525.

[10] Weinglass, A. R., Frame, E. G., and Williams, R. H., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 216.

[11] Lazarow, A., and Palay, S. L., in press, 1946.

enzymes inactivated by alloxan.

Alloxan can react directly with cysteine to form dialuric acid,[16,15] which is probably not diabetogenic.[17,15] Bruckman and Wertheimer[18] reported that a "super-saturated" solution of dialuric acid injected intravenously into rats did produce diabetes, but, since dialuric acid is rapidly converted to alloxan on standing in air,[15] their results might be due to alloxan contamination.

Ascorbic acid is also reported as capable of reducing alloxan to dialuric acid.[19] If the mechanism of sulfhydryl protection were simply reduction of alloxan to a nondiabetogenic compound, dialuric acid, one would also expect ascorbic acid to protect against alloxan diabetes. Ascorbic acid does not protect against alloxan diabetes, but rather appears to intensify the severity of the diabetes, for the 48-hour blood sugars are considerably higher in the animals given ascorbic acid plus alloxan than are those of the rats given alloxan alone (610 mg per 100 cc compared with 397 mg per 100 cc). However, interactions of ascorbic acid and alloxan within the body may not occur as readily as they do *in vitro*. Since alloxan exerts its diabetogenic effect within the first few minutes of injection,[9,20] the relative rates of interreaction of alloxan with cysteine or with ascorbic acid might influence their protective abilities.

If the administered sulfhydryl groups were able to reactivate the enzymes inactivated by alloxan, then giving the sulfhydryl compound after the administration of alloxan also should protect against diabetes. Leech and Bailey[9] have shown that alloxan disappears from the blood within 2 minutes following its intravenous injection. Gomori and Goldner[20] found that when the vessels to the pancreas were clamped before and for 5 minutes after the injection of alloxan then the beta cells in the clamped portion of the pancreas did not undergo necrosis. This fact indicates that the alloxan had disappeared from the blood during the period of clamping. However, in the present experiments the administration of cysteine one minute after alloxan produced only partial protection, whereas, when it was given after 3 minutes following the alloxan, no significant protection resulted. Therefore, if alloxan does inactivate the SH groups of enzymes, the administered glutathione or cysteine did not reactivate them after 3 minutes had elapsed. In this connection it is interesting that succinic dehydrogenase which has been inactivated by alloxan can be only partially reactivated by glutathione.[5] The action of glutathione and cysteine, therefore, may consist of a protection of the sulfhydryl groups of the islet cells from inactivation as a consequence of the administration of large amounts of exogenous sulfhydryl compounds. Although this protection may simply be due to direct inactivation of alloxan by the administered SH groups, a more complex mechanism may also be involved.

The mechanism by which alloxan produces selective degeneration of the beta cells is not understood. Although other cells (kidney, liver, and occasionally, adrenal) may, at times, show degeneration following alloxan administration, the beta cells of the islets are by far the most sensitive to alloxan. Since the beta cells are highly specialized cells, for their main function apparently consists of the synthesis and secretion of insulin, their enzyme systems are probably also highly specialized. In contrast, the liver and kidney cells which do many things may have a number of alternate enzyme pathways. If the enzyme systems of the highly specialized beta cells require active sulfhydryl groups, whereas the less specialized cells of the body possessed alternate nonsulfhydryl pathways, the apparent selectivity of alloxan for the beta cells might be explained. However, this hypothesis could not easily explain the occasional occurrence of necrosis in the kidney or liver after the administration of

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[17] Goldner, M. G., and Gomori, G., *Endocrinology*, 1944, **35**, 241.

[18] Brückman, G., and Wertheimer, E., *Nature*, 1945, **155**, 267.

[19] Ruben, J. A., and Tipson, R. S., *Science*, 1945, **101**, 536.

[20] Gomori, G., and Goldner, M. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 232.

alloxan.

Following the injection of alloxan into animals, there is a rapid decrease in the glutathione content of the body. DeCaro and Rovida[21] reported that within 10 minutes following the injection of alloxan into rats (200 mg/kg i.p.) the glutathione content of the liver fell to 17% while that of the intestine fell to 42% of their normal values. Leech and Bailey[9] reported that following the injection of alloxan into rabbits (200 mg/kg i.v.) the blood glutathione dropped rapidly in some cases to almost zero. Within 5 minutes after the injection the blood glutathione values began to rise and returned to normal by 18 to 24 hours.

Because injected alloxan causes a decrease in the glutathione content of the tissues, and because glutathione protected against alloxan damage, it is possible that the amount of glutathione present within the cell may determine its resistance to alloxan. If the beta cells of the islets of Langerhans contained less glutathione than other cells of the body, their greater susceptibility to alloxan might thus be explained.

Various species have been reported to have different susceptibilities to alloxan. Hooded rats, for example, are more resistant to alloxan than are albino rats.[22] Whereas a rat becomes diabetic after a dose of 40 mg/kg i.v., human beings can tolerate much larger doses without showing any effect.[23,24] However, a single dose of alloxan (600 mg/kg i.v.) did produce an effect on the blood sugar and islets of humans.[24] Variations in species susceptibility to alloxan might also be explained, in part at least, by differences in the amount of glutathione or cysteine they contain.

The question of whether alloxan may be involved in human diabetes is an interesting one.

1. Theoretically, at least, alloxan could be formed from uric acid, a normal metabolite. Under certain conditions an enzyme present in dog liver can convert uric acid into dialuric acid.[25] Dialuric acid is rapidly oxidized to alloxan by air. This latter reaction, however, may not occur as readily within the body, for the oxidation of dialuric acid to alloxan is inhibited by protein.[26] Studies of the possible formation of alloxan from uric acid within the human body need to be undertaken.

2. The changes seen in the pancreas of rats which have been given alloxan and have been diabetic for many months are not greatly different from those seen in human diabetes. Although some investigators have stated that the cytological changes of the pancreas in alloxan diabetes are quite different from those occurring in human diabetes, they have usually compared the lesions seen in human diabetics with those occurring in the acute stages following alloxan administration.[27] The islets of diabetic rats 5 to 7 months after the injection of alloxan appeared essentially normal when examined with the usual histological stains (hematoxylin and eosin). However, when they were examined by special granule stains, they showed a marked decrease in the number of beta cells present.[28] Similarly, the islets of human diabetics often appear normal when examined by the ordinary histological methods. It is doubtful whether the hyalinization seen in some cases of human diabetes is significant for it is not always present and may even be present in the absence of diabetes.[29] However, in 3 of 5 human diabetics whose islets

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[22] Duff, G. L., and Starr, H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 280.

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[27] Duff, G. L., *Am. J. Med. Sci.*, 1945, **210**, 381.

[28] Lazarow, A., and Palay, S. L., unpublished observations.

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