The Glutathione Content of Blood and Tissues Following Alloxan and Dehydroascorbic Acid Injections

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The mechanism of the production of diabetes in animals given injections of alloxan has been the subject of much study in recent years. Since the original publication of Dunn, Sheehan & McLetchie (1943) it has been established that the diabetes is pancreatic in origin but the chemical mechanisms involved have remained in doubt. Leech & Bailey (1945), using the arsenophosphotungstic acid method, were among the first to observe a precipitous but temporary fall in the concentration of glutathione in the blood of rabbits after the intravenous injection of diabetogenic amounts of alloxan. Since then other workers (Bruckmann & Wertheimer, 1947; Binet, Wellers & Marquis, 1949; Collin-Williams, Renold & Marble, 1950) have reported similar falls in glutathione in the blood of rats and guinea pigs after alloxan injection, though the guinea pigs do not develop diabetes (West & Highet, 1948). On the basis of these observations upon blood glutathione concentration and other circumstantial evidence, Lazarow (1949) suggested that alloxan forms an addition compound with sulphhydryl groups in the β cells of the islets of Langerhans, so causing death of these cells.

Certain structural similarities between dehydroascorbic acid (DHA) and alloxan suggested that the former compound might also be diabetogenic. Patterson (1949, 1950) was the first to report the production of diabetes in rats given large amounts of DHA by intravenous injection. Lazarow (1954) in particular has suggested that the mechanism of the production of diabetes by DHA may be similar to that by alloxan and cites evidence in support of this view. Measurements have therefore been made, and are recorded in this paper, of the concentration of glutathione in the blood and tissues of rats and in the blood of rabbits following the intravenous injection of alloxan and DHA. In the former animals both substances are diabetogenic and produce histological changes in the islet cells of the pancreas (MacDonald & Bhattacharya, 1956). Since, in these experiments, the specific glyoxalase method of Dohan & Woodward (1939), as modified by Bhattacharya, Robson & Stewart (1955) for the determination of glutathione (GSH), oxidized glutathione (GSSG) and total glutathione

(GSH + GSSG), was employed, opportunity was taken to repeat the experiments of Leech & Bailey with alloxan and to extend the observations to include tissue analyses.

METHODS

Animals and diets used. Adult rabbits of both sexes weighing 1500–3000 g. and Wistar albino rats (Glaxo) of both sexes and 125–310 g. in weight were used in the experiments.

Because of the reported influence of diet upon susceptibility to experimental diabetes (Kass & Waisbren, 1945; Grunert & Phillips, 1949) details of the diets employed in these experiments are given in full. The rabbits were maintained on pellets of the following composition: bran 15%, barley meal 20%, ground-nut meal 15%, linseed cake 10%, dried meat and bone meal 8%, dried grass meal 30%, CaCO_3 1% and NaCl 1%. The crude digestible protein of this diet was 16.5%, fat 4.6%, soluble carbohydrate 33.7% and fibre 6.7%. Each rabbit received approximately 100 g. of the pellets/day, about 15 g. of hay/day and water *ad lib*. Supplements of cabbage were given twice weekly. The rats were fed on rat cubes of the following composition: wheat offal bran 17.7%, ground wheat 17.7%, Sussex ground oats 17.7%, ground maize 8.8%, meat and bone meal 8.8%, ground barley 8.8%, fish meal 4.5%, dried skimmed milk powder 14%, NaCl 0.4%, cod-liver oil 0.4%, dried yeast 1.2%. The rats had free access to water and food.

Collection of blood and preparation of tissues. Blood was withdrawn by cardiac puncture from rabbits without sedation and from the abdominal aorta of rats, under nembutal anaesthesia. Heparin was used as anticoagulant. The blood (3 ml.) was immediately added slowly and with shaking to 3% (w/v) sulphosalicylic acid (12 ml.). In a few preliminary experiments the rabbits were sedated with nembutal for cardiac puncture and injection of DHA but later this was discontinued in view of the high death rate in such animals when injected with DHA. The rats were injected with DHA without any anaesthesia, unless otherwise stated. For the collection of tissues, however, the rats were always anaesthetized with nembutal. For tissue analysis a suitable amount was added to 5–7 ml. of sulphosalicylic acid and weighed. It was then ground with acid-washed sand, the supernatant solution being poured into a volumetric flask. The grinding with acid was repeated several times, and the combined extracts were made up to a standard volume with sulphosalicylic acid. Tissue weights are given as wet weights.

Solutions for injection. DHA solution (100 mg./ml.) in 0.9% NaCl was prepared using the method of Patterson (1950). Since this solution contains a trace of quinol as indicated by the Chloramine-T test, control animals were injected with ether-extracted solutions of pure quinol in normal saline, the ether being removed in both test and control solutions by suction. Since in the preparation of DHA, 0.66 g. of quinone is reduced by 1.08 g. of ascorbic acid, the control solutions were made by dissolving 0.66 g. of quinol in 10 ml. of ether and shaking with 10 ml. of NaCl (0.9 g./100 ml.) for 15 min. Five further ether extractions of the saline were then made as in the preparation of DHA. The DHA solutions freshly prepared in this way were found to be reducible (95–98%) by H_2S at pH 3.5 and 37° (Levenson, Rosen & Hitchings, 1951).

10% (w/v) alloxan (Genatosan Ltd.) solution was prepared in 0.9% NaCl immediately before use.

Injection method. Injections of alloxan or of DHA were given into the marginal ear vein of the rabbits and into the tail vein of the rats. In the case of DHA an initial desensitizing dose (D.D.) of 200–500 mg. was given to rabbits and 30 mg./100 g. body weight to rats 15 min. before the administration of the final dose (F.D.). In some rabbits the latter was given in divided amounts over a period of 60 min. to several days. Details of dosage used in each experiment are given later.

Chemical methods. GSH in tissues and blood was determined using the modified glyoxalase method as reported by Bhattacharya *et al.* (1955), 3% (w/v) sulphosalicylic acid being used as the protein precipitant. The validity of the glyoxalase method in the presence of amounts of DHA from 0.125 to 1.0 mg. with 0.05 mg. of pure GSH was tested. The glyoxalase reaction remained unaffected.

Oxidized glutathione in the blood filtrates was reduced to GSH electrolytically and the total glutathione was then measured by the glyoxalase method, using the technique of Dohan & Woodward (1939) as modified by Bhattacharya *et al.* (1955). In a few instances glutathione was determined

in blood by the iodometric method of Woodward & Fry (1932) or Potter & Franke's (1935) modification of Benedict & Gottschall's (1933) technique.

Blood glucose was determined by the method of Hagedorn & Jensen (1923*a, b*) at least 24 hr. after the injection of DHA.

RESULTS

Effect of alloxan injection on blood glutathione in rabbits

Table 1 shows the results of the determination of blood glutathione in rabbits following a single intravenous injection of 20 mg./100 g. body weight of alloxan. Blood samples were withdrawn immediately before (control) and immediately after the injection (<1 min.) and in some instances 15 and 60 min. after the injection. The table includes figures for pre-existing blood GSH and total GSH after electrolytic reduction, using the glyoxalase method, and also figures for pre-existing GSH, using the method of Benedict & Gottschall (1933) as modified by Potter & Franke (1935), since this was the method employed by Leech & Bailey (1945).

Although the two methods do not give the same result in the blood samples withdrawn before the injection of alloxan, both show a precipitous fall in the concentration of GSH after the injection. The average fall in the blood concentration of GSH immediately following the injection using the glyoxalase method is 23.6 mg./100 ml. In the one animal in which the determination was made 60 min. after the injection, the concentration was found to have returned almost to the pre-injection value. These results confirm those obtained by

Table 1. *Concentration of GSH, GSSG and total GSH in rabbit blood before and after intravenous injection of alloxan (20 mg./100 g. body weight)*

Weight of rabbit (kg.)	Time of blood withdrawal (min.)	Packed red blood cell volume (%)	Glyoxalase method			Potter & Franke's modification of Benedict & Gottschall's method. GSH (mg./100 ml.)
			GSH (mg./100 ml.)	GSSG (mg./100 ml.)	Total glutathione (mg./100 ml.)	
1.5	Control	44	53	20	73	70
	<1	42	18	28	46	32
	15	—	—	—	—	28
1.6	Control	42	55	13	68	70
	<1	42	23	3	26	32
2.1	Control	40	20	11	31	—
	15	39	9	16	25	—
3.0	Control	42	24	16	40	—
	<1	43	4	27	31	—
2.5	Control	48	25	21	46	—
	<1	51	6	22	28	—
	60	43	21	17	38	—
1.6	Control	40	30	13	43	—
	<1	45	18	6	24	—
Average change immediately (1 min.) after alloxan injection		—	-23.6	+0.6	-23.0	—

Leech & Bailey, and the similarity of the results obtained by the two methods along with the specificity which may be attached to one of them support the view that there is an immediate and temporary fall in blood GSH concentrations after alloxan injection. The concentrations of GSH and GSSG in these blood samples, using the glyoxalase procedure after electrolytic reduction of the filtrate (total GSH), are also given. Total GSH figures obtained immediately after injection are consistently smaller than those obtained before the injection, the average reduction being 23.0 mg./100 ml. It may be deduced from this that the diminution in the concentration of GSH in the blood is not solely or even largely due to oxidation to the GSSG form, although in some cases such oxidation seems to have occurred to some extent and the concentration of GSSG has risen. The results are consistent with the formation of a complex between GSH and alloxan for which *in vitro* evidence has been advanced by Lazarow, Patterson & Levey (1948) and Patterson, Lazarow & Levey (1949). Animals of comparable weight given control injections of saline showed no alterations in the concentration of GSH in the blood.

Effect of alloxan injection upon the glutathione content of rat blood and tissues

Table 2 shows the concentrations of glutathione, using the glyoxalase method, found in the blood, liver and pancreas in five rats given a single intravenous injection of alloxan 20 mg./100 g. body weight. Blood and tissue were taken and analysed at times following injection varying from im-

mediately after the completion of the injection to 15 min. after the administration of alloxan. Table 3 gives corresponding figures for control rats given a single injection of saline intravenously. The figures confirm the reduction in the concentration of GSH in the blood of rats reported by Bruckmann & Wertheimer (1947). That this decrease in the blood is the result of either destruction or complex formation is again seen from the values obtained for total glutathione after electrolytic reduction. There is also a striking reduction in the concentration of GSH in the liver without the appearance of appreciable amounts of GSSG, the figures obtained in the alloxan-injected animals being smaller than, and showing no overlap with, the figures of the control group. There is no apparent difference in the values obtained for GSH in the pancreas of the control group as compared with those obtained in the alloxan-injected animals.

Effect of dehydroascorbic acid upon blood glutathione in rabbits

Tables 4 and 5 give the concentrations of blood glutathione of rabbits before the injection of a desensitizing dose of DHA (control) and at intervals after a final dose of DHA given 15 min. later. The amounts of DHA injected are shown in the tables. In a few animals the final dose was not given as a single injection, but in parts at 10–15 min. intervals. Table 4 shows values of blood GSH, total glutathione and GSSG determined by the iodometric method of Woodward & Fry (1932). It has already been established that the GSH values obtained by this method, when applied to blood,

Table 2. Concentration of GSH, GSSG and total GSH (glyoxalase method) in rat blood and tissues at various intervals following intravenous injection of alloxan (20 mg./100 g. body weight)

Time after injection (min.)	Blood			Time after injection (min.)	Liver			Time after injection (min.)	GSH (mg./100 g.)
	GSH (mg./100 ml.)	GSSG (mg./100 ml.)	Total glutathione (mg./100 ml.)		GSH (mg./100 g.)	GSSG (mg./100 g.)	Total glutathione (mg./100 g.)		
<1	13	17	30	<1	192	—	—	<1	57
<1	11	5	16	<1	172	0	164	<1	42
5	4	—	—	7	126	—	—	10	56
10	3	0	3	12	223	9	232	15	64
10	4	5	9	12	151	6	157	15	29
Mean	7.0				172.8				49.6

Table 3. Concentration of GSH, GSSG and total glutathione (glyoxalase method) in rat blood and tissues following intravenous injections of saline

No. of rats	Constituent	Blood (mg./100 ml.)	Liver (mg./100 g.)	Pancreas (mg./100 g.)
5	GSH	21 (18–23)	252 (238–264)	51 (42–63)
2	GSSG	19 and 22	0	0
2	Total glutathione	41 and 40	245 and 250	50

do not differ significantly from those obtained when using the glyoxalase procedure (Bhattacharya *et al.* 1955). In contrast to the results obtained following the injection of alloxan, no consistent change occurs in the blood concentration of GSH or of total glutathione after the injection of DHA. The animals (nos. 1 and 4-6), which survived the injections of DHA, were not hyperglycaemic.

failed to reveal any significant rise in concentration. Neither animal showed any diminution in the concentration of GSH. Six other animals were starved for 12-24 hr. before the injection of DHA, 0.6 g./kg. of which was given in four equal amounts at 15 min. intervals according to the technique of Princiotto (1951). The animals failed to develop hyperglycaemia for up to 5-9 days after

Table 4. *Concentration of GSH, GSSG and total glutathione in rabbit blood as determined by the method of Woodward & Fry (1932) before (control) and at intervals following intravenous injection of DHA*

D.D. = Desensitizing dose. F.D. = Final dose.

Rabbit no.	Weight (kg.)	Time of blood withdrawal	GSH (mg./100 ml.)	GSSG (mg./100 ml.)	Total glutathione (mg./100 ml.)	Treatment given
1	2.1	Control	50	5	55	D.D. 500 mg. DHA, F.D. 1.0 g. DHA
		15 min.	47	7	54	
		40 min.	49	6	55	
		70 min.	43	10	53	
		100 min.	48	8	56	
		24 hr.	39	7	46	
		72 hr.	34	6	40	
2	1.8	Control	55	6	61	D.D. 200 mg. DHA, F.D. 800 mg. DHA
		15 min.	58	7	65	
		45 min.	58	7	65	
		75 min.	58	7	65	
3	1.5	Control	50	4	54	D.D. 500 mg. DHA, F.D. 1.0 g. DHA
		10 min.	45	9	54	
4	1.8	Control	43	4	47	D.D. 500 mg. DHA, F.D. 500-800-500 mg. DHA
		15 min.	45	10	55	
5	1.8	Control	46	4	50	D.D. 300 mg. DHA, F.D. 300-600-700-500 mg. DHA
		15 min.	48	7	55	
6	2.5	Control	47	14	61	D.D. 500 mg. DHA. Received 2 g. of DHA over 3 days
		15 min. after 3rd inj.	64	23	87	
7	2.3	Control	40	7	47	Received 20 ml. of ether- extracted quinal solution over 3 days
		15 min. after 3rd inj.	34	9	43	
8	2.4	Control	33	5	38	Received single injection of 20 ml. 0.9% NaCl
		15 min.	33	5	38	
		45 min.	33	4	37	
		75 min.	29	8	37	
		72 hr.	31	5	36	

These experiments were repeated using the specific glyoxalase method for the determination of glutathione, and the results of these are shown in Table 5. It is clear from this that in seven rabbits (nos. 1-7) there was no significant fall in the concentration of GSH in the blood following the injection of DHA. The mortality in rabbits subjected to these doses of DHA is high and this prevented observations upon the blood sugars in all but two animals (nos. 5 and 6) in which there was no significant deviation from the normal range. Two rabbits (nos. 8 and 9) were given daily intravenous injections of 500 mg. DHA for 6 consecutive days. Blood sugar determinations during the days of the injections and for 5 days following the injections

the last injection. When a further injection of DHA was given, in the manner described above, no significant alteration in blood sugar concentration was found in any animal, and the concentration of GSH in three of them (nos. 10-12, Table 5) was unaltered.

Effect of dehydroascorbic acid upon the blood and tissue concentration of glutathione in rats

Since diabetes or hyperglycaemia could not be produced in rabbits with DHA, it was decided to investigate the effects of this compound in rats which were shown by Patterson (1950) and Patterson & Lazarow (1950) to be susceptible to the diabetogenic effect of DHA.

Table 5. *Concentration of GSH, GSSG and total GSH in rabbit blood as determined by the glyoxalase method before and at intervals following the intravenous injection of DHA*

D.D. = Desensitizing dose. F.D. = Final dose.

Rabbit no.	Weight (g.)	Time of blood withdrawal	GSH (mg./100 ml.)	GSSG (mg./100 ml.)	Total glutathione (mg./100 ml.)	Packed red blood cell volume (%)	Treatment
1	1.8	Control	24	5	29	—	D.D. 200 mg. DHA, F.D. 1.5 g. DHA
		15 min.	25	4	29	—	
		45 min.	25	6	31	—	
2	1.85	Control	22	8	30	—	D.D. 300 mg. DHA, F.D. 1.5 g. DHA
		15 min.	24	6	30	—	
		45 min.	23	8	31	—	
3	1.75	Control	34	8	42	—	D.D. 400 mg. DHA, F.D. 1.5 g. DHA
		5 min.	39	0	39	—	
		60 min.	42	0	41	—	
4	1.8	Control	26	12	38	46	D.D. 400 mg. DHA, F.D. 1.3 g. DHA
		<1 min.	25	13	38	45	
		30 min.	27	10	37	43	
5	1.5	Control	39	7	46	41	D.D. 400 mg. DHA, F.D. 800 mg. DHA
		<1 min.	41	3	44	43	
		15 min.	39	5	44	42	
6	2.8	Control	42	17	59	44	D.D. 500 mg. DHA, F.D. 500–800 mg. DHA
		15 min.	48	18	66	46	
7	2.1	Control	17	11	28	40	D.D. 400 mg. DHA, F.D. 1.2 g. DHA. Received 1.8 g. of DHA over 2 days
		<1 min.	19	7	26	42	
		20 min.	17	10	27	40	
		Before 3rd inj.	30	14	44	40	
		<1 min. after 3rd inj.	29	15	44	40	
		30 min. after 3rd inj.	36	6	42	38	
8	2.0	Control	23	6	29	38	D.D. 200 mg. DHA. Received 500 mg. DHA daily for 6 days
		24 hr. after last inj.	29	7	36	36	
9	2.8	Control	26	11	37	45	D.D. 200 mg. DHA. Received 500 mg. DHA daily for 6 days
		24 hr. after last inj.	27	11	38	40	
10	1.6	Control	35	13	48	40	(a) After 12 hr. fasting received 0.6 g./kg. of DHA in 4 equal amounts at 15 min. intervals
		4 days after 2nd inj.	34	12	46	38	(b) The same treatment was repeated 5 days later after 24 hr. fasting
11	1.8	Control	43	11	54	43	(a) The same as above (b) The same as above
		4 days after 2nd inj.	34	14	48	38	
12	2.7	Control	55	19	74	42	After 24 hr. fasting received 0.6 g./kg. DHA in 4 equal amounts at 15 min. intervals
		2 hr. after inj.	62	19	81	45	

Tables 6 and 7 give the results obtained in these experiments. Following a desensitizing dose of 0.3 ml. (100 mg./ml.) DHA solution/100 g. body weight the rats were given 0.7–0.8 ml. of the same solution/100 g. body weight daily for 3 consecutive days. Control animals were given the same amount and number of injections of ether-extracted quinol solution, both groups being fasted for 12 hr. before the first injection. From 4 to 10 days after the last injection the animals were killed and blood and tissues taken for analyses. All the rats given DHA

became diabetic, and, as Table 6 shows, their fasting blood sugar concentrations ranged from 235 to 500 mg./100 ml. The control animals did not show any change in blood sugar. The mean concentration of blood GSH in the DHA-injected group was not significantly different from the control. Furthermore, analysis of liver and pancreas failed to show any appreciable difference in the GSH concentration in the control and diabetic group. The GSSG in the blood of diabetic rats was not increased and GSSG did not appear in the tissues.

Table 6. *Concentration of GSH and GSSG determined with the glyoxalase method in blood and tissues of rats 4-10 days after the last of three daily injections of ether-extracted quinol solution (controls) and of DHA, a desensitizing dose of the latter being also given*

For details of dosage see text.

Control rats			DHA-diabetic rats			
Blood			Blood			
Rat no.	GSH (mg./100 ml.)	GSSG (mg./100 ml.)	Rat no.	Blood sugar after injection (mg./100 ml.)	GSH (mg./100 ml.)	GSSG (mg./100 ml.)
11	18	26	17	333	21	25
12	18	24	18	336	19	16
13	21	23	19	461	26	16
14	23	22	23	436	24	15
15	24	24	27	500	23	17
16	20	18	28	235	18	20
17	18	20				
18	15	21				
19	19	25				
20	18	20				
Mean	19.4	22.3	Mean		21.8	18.1
Liver			Liver			
	(mg./100 g.)	(mg./100 g.)		(mg./100 ml.)	(mg./100 g.)	(mg./100 g.)
1	136	—	3	378	180	0
2	193	0	4	416	215	0
3	232	—	6	392	165	1
4	242	—	7	423	218	0
5	148	—	8	423	237	35
6	230	0	9	392	188	2
7	225	—	13	428	205	—
8	201	—	16	427	242	—
9	175	7				
10	199	—				
Mean	198.1		Mean		206.2	
Pancreas			Pancreas			
	(mg./100 g.)	(mg./100 g.)		(mg./100 ml.)	(mg./100 g.)	(mg./100 g.)
3	48	0	4	416	74	0
4	56	0	8	423	85	0
7	58	0	9	392	53	0
8	63	—	12	349	49	0
Mean	56.2		Mean		65.2	

In view of the rapidity of the changes in the concentration of GSH following alloxan injection, observations were made in blood and tissues 10-17 min. following the last injection of DHA given on the third day, the previous daily injections, including the desensitizing dose, being given as previously described. The results of these experiments are shown with the appropriate controls in Table 7. As before, there is no significant alteration in the blood concentration of GSH or GSSG. In the liver, however, the figures indicate an average reduction in GSH of 60.3 mg./100 g. without appearance of any GSSG. This difference is statistically significant ($P < 0.01$). This reduction in liver GSH is in contrast with the findings reported in Table 6; in these experiments, however, the liver samples were obtained 4-10 days after the

last injection of DHA. Analyses of GSH in pancreas show that there has been an average increase in GSH concentration of 16.2 mg./100 g. tissue.

Immediate effect of the injection of alloxan or dehydroascorbic acid upon the concentration of glutathione in the liver of rats

In the above experiments estimations of glutathione in the liver were performed after three daily injections of DHA. In view of the rapidity and the temporary nature of the alterations in blood glutathione concentration following alloxan injection it was thought desirable to repeat the analysis of liver glutathione immediately following the first main injection of DHA. The following experiments were therefore performed. Under

nembutal anaesthesia a suitable amount (one small lobe, approx. 0.5–0.9 g.) of liver of the rat was removed for glutathione analysis, bleeding being controlled by mosquito haemostats and cotton wool. The animals were then given an intravenous injection of (a) 25 mg. DHA/100 g. body weight

as a desensitizing dose followed by 55–140 mg. DHA/100 g. 15 min. later, or (b) ether-extracted quinol solution in 0.9 % NaCl solution or (c) 20 mg. alloxan/100 g. body weight. 15–30 min. after the conclusion of injection another sample of liver tissue was taken for analyses.

Table 7. *Concentrations of sugar, and GSH and GSSG obtained with the glyoxalase method, in blood and tissues of rats, 10–17 min. following the last injection of three daily injections of ether-extracted quinol solution (controls) and of DHA, a desensitizing dose of the latter being given*

For details of dosage see text.										
Blood				Liver				Pancreas		
Rat no.	Blood sugar before 3rd injection (mg./100 ml.)	GSH (mg./100 ml.)	GSSG (mg./100 ml.)	Rat no.	Blood sugar before 3rd injection (mg./100 ml.)	GSH (mg./100 g.)	GSSG (mg./100 g.)	Rat no.	Blood sugar before 3rd injection (mg./100 ml.)	GSH (mg./100 g.)
Control rats										
1	124	16	24	1	124	221	0	1	124	54
3	108	16	20	2	114	220	—	2	114	63
4	101	16	22	3	108	205	—	3	108	60
5	117	15	21	4	101	241	0	4	101	62
6	113	17	20	5	117	202	—	5	117	60
7	—	19	20	6	113	209	—	6	113	54
Mean		16.5	21.1	—	—	216.3	—	—	—	58.8
DHA-injected rats										
1	402	19	23	1	402	231	0	1	402	97
7	363	17	21	2	—	148	4	2	—	53
8	214	19	17	7	363	132	0	7	363	103
9	328	14	17	8	214	138	0	8	214	70
10	230	16	20	9	328	119	—	9	328	68
11	122	19	19	10	230	168	—	10	230	61
								11	122	73
Mean	—	17.3	19.5	—	—	156.0	—	—	—	75.0

Table 8. *Concentration of liver GSH, as determined by the glyoxalase method before and 15–30 min. after intravenous injection of alloxan, DHA and ether-extracted quinol solution in saline*

Rat wt. (g.)	Solution given	Total dose	GSH before injection (mg./100 g.)	GSH after injection (mg./100 g.)	Δ (mg./100 g.)	Time after injection (min.)
250	DHA	165 mg./100 g.	295	200	–95	30
125		80 mg./100 g.	341	290	–51	30
205		85 mg./100 g.	229	190	–39	30
220		100 mg./100 g.	271	159	–112	30
240		100 mg./100 g.	243	151	–92	30
				Mean	–77.8	
310	Alloxan	20 mg./100 g.	322	94	–228	15
170		20 mg./100 g.	254	149	–105	15
200		20 mg./100 g.	252	68	–184	30
230		20 mg./100 g.	202	74	–128	30
				Mean	–161.2	
250	Saline	4.0 ml.	329	337	+8	30
145		4.0 ml.	214	214	0	30
180		0.5 ml.	284	270	–14	15
190		0.5 ml.	190	161	–29	30
190		0.5 ml.	221	226	+5	30
200		0.5 ml.	266	259	–7	30
205		0.5 ml.	216	228	+12	30
				Mean	–3.5	

The results of these experiments are shown in Table 8. The animals given DHA or alloxan invariably show a considerable fall in the concentration of GSH after the injection. The decrease is less marked in the DHA-injected animals (average 77.8 mg./100 g. tissue) as compared with the decrease (av. 161.2 mg./100 g. tissue) in those given alloxan. No significant decrease in the GSH concentration follows the administration of ether-extracted quinol solution. In two animals given alloxan and in two animals injected with DHA, total glutathione determined after electrolytic reduction of the tissue extract shows that the decrease in GSH is not due mainly to simple oxidation (Table 9). Only a small proportion of the amount of GSH which disappeared from the liver is recoverable by this procedure.

reduction suggest that the fall in blood glutathione is not solely or even largely due to oxidation of GSH to GSSG, since this procedure does not result in regeneration of the original glutathione concentration. Although the present data offer no direct evidence in support of the view that a complex of alloxan and GSH is formed *in vivo* following the injection of alloxan, the results we have obtained are consistent with that view.

The experiments on rats involving the injection of DHA confirm that this compound is diabetogenic in this species. Like Banerjee, Belavady & Mukherjee (1953) we have been unable to render rabbits diabetic with intravenous injections of DHA. This negative result has occurred even when the injection technique of Princiotto (1951) is used and, in addition, we have employed repeated

Table 9. *Concentration of GSH, GSSG and total glutathione, as determined by the glyoxalase method, in rat liver before and 30 min. after injection of DHA and alloxan*

The rats were given 20 mg. of alloxan/100 g. body wt. or 100 mg. of DHA/100 g. body wt.

Rat no.	Before injection			After injection (1) of alloxan		
	GSH (mg./100 g.)	GSSG (mg./100 g.)	Total glutathione (mg./100 g.)	GSH (mg./100 g.)	GSSG (mg./100 g.)	Total glutathione (mg./100 g.)
1	271	5	276	62	27	89
2	288	6	294	87	24	112
(2) of DHA						
1	251	0	243	108	15	123
2	271	0	271	119	13	132

DISCUSSION

The above results confirm the observation that following the intravenous injection of diabetogenic amounts of alloxan into rabbits and rats there is a rapid and quickly reversed decrease in the concentration of GSH in the blood (Leech & Bailey, 1945; Bruckmann & Wertheimer, 1947). The high specificity of the method we have employed for glutathione determination strengthens the conclusion, and the extension of the analyses to the tissues of rats demonstrates that this decrease in concentration occurs also in the liver.

The cause of the fall in the blood and hepatic concentration of glutathione following alloxan injection remains uncertain. The affinity of alloxan for sulphhydryl compounds has been emphasized by Lazarow *et al.* (1948) and Patterson *et al.* (1949). They showed *in vitro* that an alloxan-GSH complex possessing a peak ultraviolet absorption at 305 m μ . rapidly forms when the two compounds are mixed together in aqueous solution at physiological pH. We have confirmed this observation. The figures obtained for total blood GSH after electrolytic

injections of DHA without success. We can offer no explanation to account for the difference between Princiotto's results and those obtained by Banerjee *et al.* and by ourselves.

The failure to produce diabetes in rabbits accompanied the finding that no significant alteration in blood glutathione occurred following the injections. In this respect it is surprising that Banerjee *et al.* observed a fall in the concentration of GSH in rabbits following the injection of DHA, since they employed a method, i.e. iodometric titration (Woodward & Fry, 1932), with which high concentrations of ascorbic acid interfere. In the course of this work it has been found that the latter compound was present in the plasma in very high concentration for some time after the injection of DHA in the amounts used.

The GSH concentration in the pancreas of rats immediately following the intravenous injection of diabetogenic doses of alloxan or DHA did not decrease. In the pancreas of rats made permanently hyperglycaemic with DHA the concentration of GSH remained almost unaltered. These findings are apparently contradictory to the sulphhydryl

theory of Lazarow (1949, 1954); we agree, however, with Lazarow (1949) that very little information is given by the GSH analysis of the whole pancreas regarding the GSH content of the β -cells in the islets of Langerhans of the pancreas, because the β -cells constitute only a fraction (0.5%) of the total weight of the organ.

The acute experiments in which estimations of liver glutathione were made following the injection of alloxan, DHA and ether-extracted quinol solutions suggest that the different response to alloxan and DHA injections is quantitative rather than qualitative. In these experiments, and in spite of there being no alteration in blood glutathione concentration, the liver concentration of glutathione fell significantly in animals given injections of DHA, although the changes were smaller than those which followed injections of alloxan. Nevertheless, the implication of the results is that both diabetogenic agents are associated with a fall in the body content of glutathione.

There is no evidence that GSH is converted into GSSG in significant quantities, and what happens to the GSH which disappears from the liver after DHA injection is still unknown. This is surprising in view of the fact that part of the injected DHA is reduced to ascorbic acid—a process believed to involve GSH as a hydrogen donor (Borsook, Davenport, Jeffreys & Warner, 1937; Schultze, Stotz & King, 1938; Thomson, unpublished). The *in vitro* experiments of Drake, Smythe & King (1942) involving polaroscopic analysis, provide evidence that at pH 2–3.5 there is a reaction between DHA and GSH. We have repeated and confirmed these observations, but their physiological relevance is open to question since it is not possible to demonstrate the reaction at a physiological pH. In aqueous solution at pH 7.4 and in the presence of GSH, DHA rapidly becomes converted into dioxogulonic acid. The fact, however, that electrolytic reduction of the liver extracts after injection of DHA does not result in regeneration of GSH is evidence that the diminution in the hepatic concentration is not solely or even largely due to simple oxidation.

SUMMARY

1. After the intravenous injection of diabetogenic amounts of alloxan, the concentrations of glutathione (GSH) and total glutathione (GSH + oxidized glutathione) are greatly decreased in the blood of rabbits and rats.

2. Following the intravenous injection of diabetogenic doses of alloxan into rats, the GSH concentration in the liver shows a precipitous fall, but that of the pancreas remains practically unchanged. Oxidized glutathione, which is not present in the

liver of normal rats, does not appear in this tissue in significant amounts after such injection.

3. We have failed to produce hyperglycaemia in rabbits given large doses of dehydroascorbic acid by intravenous injection. In contrast, hyperglycaemia has been regularly produced in rats given dehydroascorbic acid by intravenous injection.

4. When dehydroascorbic acid is injected intravenously into rabbits, the concentration of GSH, oxidized glutathione and total glutathione in the blood does not change significantly.

5. In rats made permanently hyperglycaemic with dehydroascorbic acid the concentration of GSH in the blood, liver and pancreas is not altered; the content of oxidized glutathione of the blood remains the same and it does not appear in significant amounts in liver and pancreas.

6. Immediately following the intravenous injection of diabetogenic amounts of dehydroascorbic acid to rats the blood concentration of GSH and oxidized glutathione was unaltered, but the concentration of GSH and total glutathione in the liver was markedly decreased without the appearance of oxidized glutathione. The GSH content of the pancreas showed no diminution.

7. The results indicate that the reduction in the concentrations of GSH in the blood and liver of rats and in the blood of rabbits after alloxan injection and of GSH in the liver of rats after the injection of dehydroascorbic acid is not solely or even largely due to oxidation of reduced to oxidized glutathione.

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Potentiometric and Other Studies on Preparations of Cytochrome *c* from Ox- and Horse-Heart Muscle

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Cytochrome *c* obtained from ox- and horse-heart muscle by the well-known procedure of Keilin & Hartree (1945) has an iron content of about 0.34 % (w/w) and a fairly well-defined E'_0 value, constant over a range of pH values. Treatment of this material by means of chromatography on a resin column (Paléus & Neilands, 1950; Neilands, 1952; Margoliash, 1954*a, b*) appears to provide cytochrome *c* of a purity not yet attained by other methods [0.43–0.46 % (w/w) Fe]. In the course of a study of oxidation–reduction potentials of various metallo-porphyrin systems by potentiometric titration, it seemed of interest to compare the E'_0 value of this highly purified cytochrome *c* with that of the '0.34 %' iron preparation.

While we were preparing the cytochrome, our attention was drawn to the need for closer investigation of the methods of dialysis, by the observation that a non-cytochrome, haem-protein precipitate, present in copious amounts after dialysis of ox-heart preparations against sodium chloride, was not found after dialysis against ammonium hydroxide.

This observation, which does not seem to have been reported previously, seemed to be of importance, as it was obvious that the impurity was incorporated into cytochrome preparations when using ammonium hydroxide; this procedure is now common for preventing loss of cytochrome through the dialysis sac, and has been accepted as a standard alternative to the procedure of Keilin & Hartree (1952).

Details are given of the potentiometric titration apparatus. These include the nitrogen purification system which is designed to take into account the often-neglected finding of Keilin & Hartree (1943) that oxides of nitrogen may be generated by passage of nitrogen over heated copper. Other trace gases which interfere with many metallo-porphyrin systems are also eliminated in the purification system used.

MATERIALS AND METHODS

Cytochrome c preparations

Samples with a '0.34 % iron content'

Fresh ox or horse hearts were treated usually in 2–3 kg. batches according to the method of Keilin & Hartree (1945). The preparations were then dialysed against either 0.5 % (w/v) NaCl or 0.01 N-NH₄OH. In two cases, preparations (c) and (d), the material was divided after the final washing with saturated (NH₄)₂SO₄; one half of each was dialysed against 0.5 % (w/v) NaCl and the other against 0.01 N-NH₄OH. All dialyses were carried out in Visking seamless cellulose tubing at 4° until sulphate-free.

The dark, non-cytochrome precipitate (*P*) left after NaCl dialysis was removed by centrifugation and the supernatant filtered to provide 'NaCl'-cytochrome *c*. The precipitate (*P*) was washed free of cytochrome *c* with 0.5 % (w/v) NaCl. The small amount of residue obtained in the case of NH₄OH dialysis was removed by filtration and discarded. The filtrate provided 'NH₄OH'-cytochrome *c*.

For testing the effect of various conditions of dialysis, one preparation was divided at the pre-dialysis stage into six portions which were then dialysed respectively against