Glutathione, Oxidized and Reduced, in the Brain and in Isolated Cerebral Tissue

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(*Received* 4 *June* 1958)

In seeking to maintain isolated cerebral tissues in a state which approximates as closely as possible to their state *in vivo*, many aspects of their chemical and metabolic constitution have been explored (see Thomas, 1956; McIlwain, Thomas & Bell, 1956). Such investigations have concerned especially the labile constituents of the tissue, known to change rapidly when blood supply ceases, and have had as objective the reversal of such changes.

Glutathione has now been examined from this point of view because it is present in considerable concentration in the brain, and can undergo rapid change there. Cerebral tissues possess a potent glutathione reductase (McIlwain & Tresize, 1957) and this led to special study, in the present work, of the oxidized and reduced forms of glutathione. Subsidiary investigations have concerned the assay of these two forms of glutathione and their determination in the brain of the rat and guinea pig rapidly fixed *in situ*.

EXPERIMENTAL

Quantities and concentrations of the oxidized form of glutathione (GSSG) are described throughout in terms of the equivalents of the reduced form (GSH) which they would yield on reduction.

Determination of glutathione

Established methods of determining the two forms of glutathione in the quantities in which they occur in small samples of cerebral tissues are not satisfactory. In 100 mg. of tissue, the quantities concerned are 0·01–0·05 μequiv. as the oxidized and 0·1–0·4 μequiv. as the reduced form. Because the existing chemical methods of determining the reduced form (see Patterson & Lazarow, 1955; Thomson & Martin, 1959) lack adequate sensitivity or specificity, we have preferred an assay based on glyoxalase. For determining oxidized glutathione, some previous investigators have combined the glyoxalase assay with a preliminary chemical or electrometric reduction of solutions to be assayed (Dohan & Woodward, 1939). In the present work, based on a loose analogy with the apozymase assay (Axelrod & Elvehjem, 1940), in which a yeast preparation is used to determine both oxidized and reduced cozymase, conditions were sought and found in which a washed yeast apoglyoxalase could itself be used to reduce the oxidized glutathione of the solutions being assayed. Francoeur & Denstedt (1954) observed that a preparation from lysed red cells, with certain additions, produced acid from methylglyoxal with either oxidized or reduced glutathione, but did not develop the system for assay. We preferred to base the present work on yeast apoglyoxalase, about which much more information is available (see Patterson & Lazarow, 1954).

Apoglyoxalase. The preparation is based on that of Patterson & Lazarow (1955); this reference should be consulted for further details. It was carried out in a room at 4° and with refrigerated reagents and equipment. Fresh baker's yeast (1 lb.; The Distillers' Co. Ltd., Croydon) was crumbled into acetone (1·5 l., A.R.) and stirred vigorously, mechanically, for 5 min. The mixture was filtered by suction, and the solid stirred similarly with 500 ml. of acetone and again sucked dry on a filter; it could be left overnight at this stage. The solid was then suspended in 1 l. of water, the suspension stirred for 5 min., and centrifuged (1500 *g*, 20 min.); the supernatant was discarded and the washing repeated six times. The final solid, well packed, was stirred with 500 ml. of acetone, and filtered and dried by suction, and the process repeated twice with acetone and once with 300 ml. of A.R. ether, after which air was sucked through the residue on the Büchner funnel for 30 min. It was then transferred to a desiccator and evacuated with a pump left running for several hours; calcium chloride and shavings of paraffin wax were added to the desiccator, which was re-evacuated and left overnight. The dried apoglyoxalase keeps in a refrigerator for several months without change in activity. A 20% (w/v) suspension in glass-distilled water was prepared immediately before use.

Glutathione. The reduced form (The Distillers' Co. Biochemicals Ltd., Liverpool) was found by iodine titration to be of 99·8% purity. The crystalline oxidized form (L. Light and Co.) was found by using glutathione reductase to be of 97–100% purity. For assay, standard solutions of each, at 0·1 and 0·2 μequiv. of GSH/ml., were prepared in 3% (w/v) sulphosalicylic acid.

Other reagents. The aqueous 30% solution of methylglyoxal supplied (L. Light and Co.) was distilled under reduced pressure (20 mm. Hg) and the viscous yellow fraction, collected in a cooled receiver, was kept at −20°. A 10% (w/v) solution in water was prepared immediately before use. $NaHCO_3$ soln. (0·8M) was equilibrated with $N_2 + CO_2$ (95:5, v/v). Sulphosalicylic acid (B.P.; 3%, w/v) was prepared in glass-distilled water.

Assay. The rate of acid formation in the glyoxalase reaction was followed manometrically (see Patterson & Lazarow, 1955). A typical determination of reduced and total glutathione in four unknown solutions involved 16

manometers with vessels which were filled as indicated in Table 1. The vessels were gassed with $N_2 + CO_2$ (95:5, v/v), and shaken in a bath at 25° and after a further 10 min. their contents mixed by tipping. After 5 min. manometric readings were commenced and taken each 5 min. for 35 min. The rate of evolution of CO_2 was obtained graphically. Standard curves were constructed, and the glutathione content of the solutions assayed was read from such curves. Fig. 1 illustrates how, in this assay, GSSG activated glyoxalase only in the vessels in which it had received prior incubation with the yeast preparation.

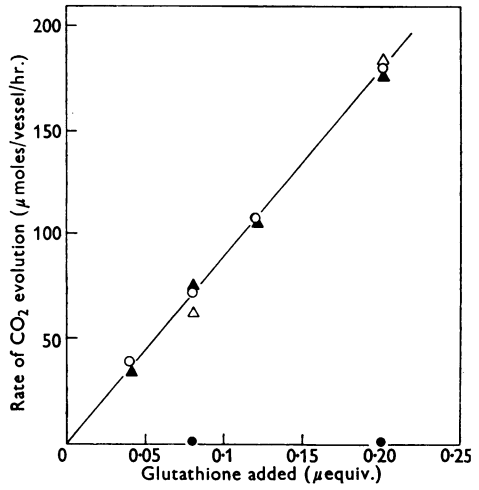

Fig. 1. Standardization of a batch of apoglyoxalase in the reaction mixture of Table 1. Rates of acid formation shown resulted from the inclusion of: ○, the oxidized and △, the reduced forms of glutathione initially in the main parts of the vessels with the apoglyoxalase; and ●, the oxidized and ▲, the reduced forms initially in the side arms.

GSH gave equal activation in the two experimental arrangements of Table 1.

Fig. 1 shows the type of experiment run with each batch of apoglyoxalase to judge its suitability for use in assay. In each individual assay, only the four standards detailed in Table 1 were included; blank values were subtracted.

Amperometric determination of glutathione. The values obtained by the glyoxalase method have been checked also by comparison with amperometric determination of glutathione, by the methods described by Thomson & Martin (1959). Close agreement has been found. Some values obtained amperometrically are included in Table 3.

Tissues

Fixation in situ. Rats and guinea pigs of less than 100 g. wt. were dropped head-first into a vacuum flask of liquid N_2. After 5 min. immersion the brain was bared with a chisel cooled in the liquid N_2 and samples of about 100 mg. were added to tared tubes of ice-cold sulphosalicylic acid.

Tissues fixed post mortem. Ox and sheep brain were removed by slaughter-house workers and placed on solid CO_2 5–10 min. after killing the animals. Guinea pigs and rats were stunned by a blow on the back of the neck and exsanguinated, the brain was removed and blocks of tissue were cut and weighed on a torsion balance. Rabbits were killed by guillotining.

Metabolic experiments. Guinea pig or rat brain was cut to slices 0·35 mm. thick, which were floated from the cutting blade to a dish of a glucose–phosphate medium containing 134 mM-NaCl, 5·2 mM-KCl, 28 mM-$CaCl_2$, 1·3 mM-$MgSO_4$, 10·4 mM-Na_2HPO_4 and 10 mM-glucose, prepared as described by Rodnight & McIlwain (1954). Then they were trimmed to weigh 70–100 mg., drained from excess of medium by repeatedly touching a glass surface and weighed. Some were fixed at this stage and others after incubation in 3·5 ml. of medium in conventional manometric apparatus. In the instances quoted, anaerobic conditions were maintained with yellow phosphorus in the centre well. Under aerobic conditions, respiration was measured and found to be of 59–65 μmoles of O_2/g. of

Table 1. *Glutathione assay: contents of vessels*

All vessels contained in the main compartment 1 ml. of 20% (w/v) apoglyoxalase and 0·025 ml. of water, after which additions were made in the order quoted in the Table. Vessels were filled and placed in a water bath at 25° in the following order: thermobarometer (2 ml. of water), GSH blank, two with GSH standard solutions, four with solutions to be assayed for GSH, four with solutions to be assayed for GSH and GSSG, two with GSSG standard solutions, the GSSG blank and a thermobarometer.

		Content (ml.) in vessels for determining			
		Reduced glutathione		Total glutathione	
Vessel compartment	Reagent	Blank	Standard or unknown	Blank	Standard or unknown
Main	0·8 M-$NaHCO_3$	0·125	0·125	0·35	0·35
	10% Methylglyoxal	0·1	0·1	0	0
	Sulphosalicylic acid	0	0	0·5	0
	GSSG standard or unknown in sulphosalicylic acid	0	0	0	0·5
Side arm	Sulphosalicylic acid	0·5	0	0	0
	GSH standard or unknown in sulphosalicylic acid	0	0·5	0	0
	0·8 M-$NaHCO_3$	0·25	0·25*	0·025	0·025
	10% Methylglyoxal	0	0	0·1	0·1

* Quantity determined by titration (see text)

Table 2. *Recovery of glutathione added in extracting cerebral tissues*

The glutathione added was contained in sulphosalicylic acid added to the tissues, which were from guinea pigs, and which were extracted as described in the text. Where appropriate, mean values are followed by S.D. and in parentheses the number of observations. Figures in brackets under GSH refer to the change in GSH values after adding GSSG, as percentage of the added GSSG.

Specimen (mg.)	Glutathione (μm.-equiv.) Added GSH	Added GSSG	Found GSH	Found Total	Recovery (% of that added) GSH	Recovery (% of that added) Total
Animal 1. Slices of cerebral cortex						
95	0	0	123	160	—	—
97	0	0	127	167	—	—
92	100	0	210	260	89	103
97	100	0	217	273	91	108
97	0	100	—	270	—	105
97	0	100	—	273	—	108
Animal 2. Slices of cerebral cortex						
107	0	0	165	230	—	—
97	0	0	160	226	—	—
96	0	40	150	260	[−10]	110
106	0	40	190	270	[+48]	80
99	0	200	170	440	[+6]	109
105	0	200	190	450	[+11]	107
Animals 1 and 2		Mean±S.D.			—	104±4 (8)
Animal 3. Ground tissue						
100	0	0	181±5 (4)	235±6 (4)	—	—
100	150	0	328±5 (4)	381±7 (4)	98±4 (4)	97±4 (4)
100	0	150	179±6 (4)	387±7 (4)	[−1±5 (4)]	102±3 (4)
Animal 4. Ground tissue						
60	0	0	—	150±3 (5)	—	—
60	0	120	—	266±5 (5)	—	97±5 (5)

tissue/hr. and unaffected by added glutathione. After chosen periods of incubation at 37° slices were removed promptly from the manometric vessels with a mounted bent wire. As glutathione had been added to the media during some experiments, all tissues were briefly (2 sec.) rinsed in a dish of medium lacking glutathione, and were then immediately extracted. A comparison of the dry weight of the original tissues and of tissues weighed at the stage described indicated that they had then absorbed fluids to the extent of 22% of their fresh weight (Rodnight & McIlwain, 1954).

Extraction. Except when stated otherwise, fixation was in sulphosalicylic acid. The acid (1·5 ml.; 3%, w/v) was contained in a homogenizer tube in ice–water, and to this samples of approx. 100 mg. of tissue were added and immediately ground with a cold pestle. The mixture was kept at 0° for 30 min. and centrifuged, and the supernatant taken for assay. Both forms of glutathione were stable in such solutions for several days. The bicarbonate of the assay mixture is partly expended in neutralizing the sulphosalicylic acid, and the amount of bicarbonate used in assay is determined by titrating the extract to pH 7. Any dilution of the extracts must be made in 3% (w/v) sulphosalicylic acid.

Recovery of added glutathione. The use of the assay for determining the oxidized and reduced forms of glutathione in cerebral extracts was checked as indicated in Table 2. When recovery was attempted (Table 2) from slices of cerebral cortex, by adding quantities of oxidized or reduced glutathione comparable with those already associated with the tissue, no loss was found. Values were, however, scattered to a greater extent than was desirable in judging recovery. This was due to variation in glutathione content from slice to slice. Recovery from a cerebral 'homogenate' (Table 2) was much more uniform and indicated no loss and no artificially high values.

RESULTS

Glutathione of tissues before metabolism in vitro

The conflicting values which have been reported for the content of oxidized glutathione in cerebral tissues received comment earlier (McIlwain & Tresize, 1957). The present observations showed that the values depended greatly on the manner of removing the tissue for analysis (Table 3). In small laboratory animals the most satisfactory method of fixation for analysis of labile cerebral constituents

appears to be by dropping animals into liquid nitrogen (see Richter & Dawson, 1948; McIlwain, 1956). Rats and guinea pigs so treated gave values for oxidized glutathione of only about 0·1 μmole/g. of tissue, or 2–5 % of the total glutathione. These low values are similar to the one result quoted for the rat by Fujita & Numata (1938).

By contrast, when these animals and also rabbits were killed by a blow or by exsanguination, and the brain was removed, weighed and ground in fixing agent approx. 3 min. after death, values for oxidized glutathione were about 10 times as high. This increase was at the expense of the reduced form: values for total glutathione did not depend on the method of sampling. In ox and sheep also, the brain frozen a few minutes after death contained an appreciable amount of oxidized glutathione. In the rabbit the quantity of oxidized form had become about a quarter of the total, a value comparable with that reported by Kudryavtseva & Kudryavtseva (1950). Examination of different parts of the brain showed the higher amount of oxidized glutathione in each part; values for total glutathione tended to be higher in grey matter than in white. In both the rat and guinea pig, concentrations of total glutathione were similar in young and adult animals (Table 3).

Metabolism in vitro *and the tissue content of glutathione*

Stunning or exsanguination, and removing the brain without freezing, is a normal preliminary to study of cell-containing cerebral tissues *in vitro*; such studies thus commence with tissues abnormally high in oxidized glutathione. Subsequent slicing and placing the tissue in a medium at room temperature was found to have little effect on glutathione concentration. In reaching this conclusion, the correction of values for swelling of the tissue by uptake of fluid, explained in the Experimental section, is to be noted.

Fig. 2 shows the fate of the tissue's glutathione when, subsequently, the tissue slices were incubated in an oxygenated glucose–phosphate medium. Results in glucose–bicarbonate medium were similar. In the phosphate medium, both oxidized and reduced forms of glutathione suffered loss. The fall in oxidized glutathione represented a change towards normal values obtained *in vivo*, but even in 2 hr. did not proceed to the low values found *in vivo*. The fall in total glutathione represented a divergence from concentrations found *in vivo*. Different conditions of incubation were therefore examined in search for circumstances tending to restore normal values.

The tissue content of total glutathione was found to be successfully maintained when glutathione itself was added to the medium. Figs. 3 and 4 show that the reduced form of glutathione was more effective in doing this than was the oxidized form, when substances were added at 0·3 mM to oxygenated glucose–phosphate media. This concentration is between those found for the plasma (0·2 mM) and whole blood (1·3 mM) of rabbit (unpublished

Table 3. *Glutathione of cerebral tissues sampled in different ways*

After sampling, as described in the Experimental section, all tissues were fixed by grinding in sulphosalicylic acid. Values are followed by standard deviation and (in the column headed Total) the number of animals sampled, except when they are the mean of only two or three results. Animals were adult except as stated.

Treatment of animal before sampling	Animal; age (days); part of brain	Glutathione (equiv. of GSH/g. of fresh tissue) Total	GSH	GSSG
Dropped into liquid N_2	Rat; 34–36 days; whole brain	3·44±0·25 (6)	3·34±0·18	0·10±0·12
	Guinea pig; 24 days; cerebral hemisphere	2·77	2·72	0·05
	Guinea pig; 3 days; whole brain	2·20 (3)*	2·12*	0·08*
Stunned; brain removed and placed in solid CO_2	Sheep; cortex	2·88	2·56	0·32
	Ox; cortex	3·71±0·04 (4)	2·90±0·23	0·81±0·20
	Ox; cerebellum	2·50±0·24 (4)	2·31±0·08	0·20±0·24
Stunned or exsanguinated; brain removed and fixed without freezing (adult animals)	Rat; cortex	3·58±0·39 (6)	2·69±0·46	0·90±0·26
	Rat; whole brain	3·43±0·12 (4)	2·92±0·18	0·51±0·32
	Rat; mid-brain	3·15	1·64	1·51
	Guinea pig; cortex	2·92±0·19 (7)	2·61±0·16	0·31±0·10
	Guinea pig; mid-brain	2·19	1·65	0·55
	Guinea pig; cerebral hemispheres	2·92±0·32 (6)	2·48±0·12	0·44±0·1
		2·70±0·16 (6)*	2·48±0·17*	0·23±0·03*
	Rabbit; cortex	3·07	2·33	0·74
	Rabbit; white matter from cerebellum	2·30	1·60	0·70

* Amperometric determination; others were by glyoxalase.

results). Nevertheless, the oxidized form of glutathione in the tissue remained high, at 0·4–0·6 μ-equiv./g., whichever form of glutathione was added to the medium. Added reduced glutathione acted more promptly than the oxidized form in restoring the tissue's reduced glutathione to normal values.

In attempting to restore the oxidized glutathione of the tissue to normal values, incubation under anaerobic conditions was examined. The absence of oxygen had, however, relatively little effect on values for either oxidized or reduced glutathione (Fig. 5). Glutathione levels proved similarly independent of the presence of glucose, in oxygenated phosphate media. Thus after incubation for 1 hr. in the absence of glucose, reduced glutathione was found at 1·69 and oxidized at 0·5 μequiv./g.; values with glucose in the same experiment were 1·36 and 0·54 μequiv./g.

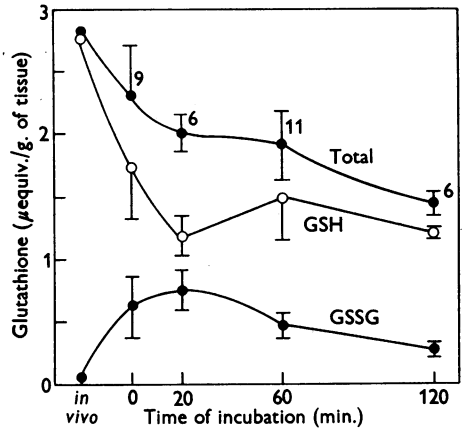

Fig. 2. Glutathione of guinea-pig cerebral cortex before and after incubation in oxygenated glucose–phosphate media. Points indicate mean values of the number of determinations quoted by the 'total' line, and vertical lines extend from points for distances equal to the standard errors of the means. The values *in vivo* were obtained after dropping the animal into liquid N_2, as described in the text.

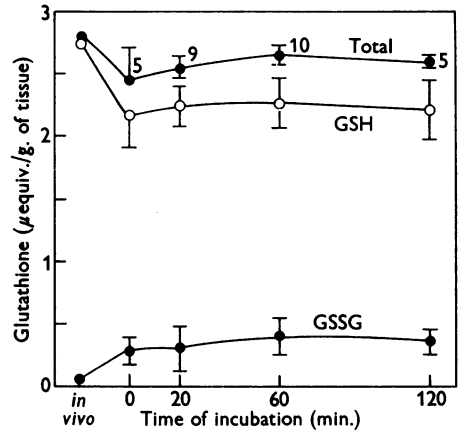

Fig. 3. Glutathione of cerebral cortex before and after incubation with 0·3 mM-GSH. Other details are as given for Fig. 2.

DISCUSSION

Comment may first be made on the present adaptation of the glyoxalase assay to determine both oxidized and reduced glutathione. As reduced glutathione is established as the coenzyme of glyoxalase and a substrate of glyoxalase I (Racker, 1951), it is understandable that the reduced rather than the oxidized form should be ordinarily determined by the yeast preparation. However,

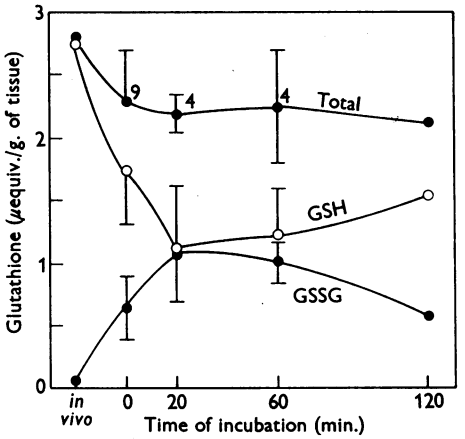

Fig. 4. Glutathione of cerebral cortex before and after incubation with 0·3 m-equiv. of GSSG/l. Other details are as given for Fig. 2.

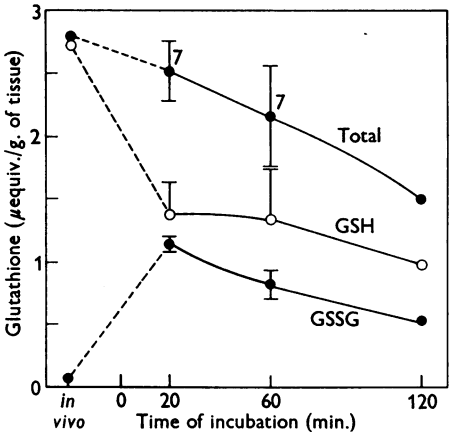

Fig. 5. Glutathione of cerebral cortex before and after incubation anaerobically. Other details are as given for Fig. 2.

although the preparation can reduce the oxidized form when incubated with it for 10 min., it is perhaps surprising that such reduction does not occur during the ordinary assay of the reduced form. In explanation it may be significant that the incubation which brings about the reduction of oxidized glutathione is of yeast plus sample to be assayed, in the absence of methylglyoxal, whereas during assay methylglyoxal is, of course, present. Methylglyoxal reacts chemically and enzymically with several thiol compounds and may do so with a thiol essential for reduction of the oxidized glutathione. Glutathione reductase has been found to be susceptible to sulphydryl reagents (Asnis, 1955).

The glutathione of cerebral tissues has proved to be one of their more stable constituents, when considered as a single component, the sum of oxidized and reduced forms. The total cerebral glutathione is not rapidly degraded on death of an animal, as is glycogen, phosphocreatine or adenosine triphosphate. Moreover, although loss occurs on incubating cerebral tissues in simple fluid media, this is readily avoided by including glutathione, preferably in the reduced form.

The balance between the oxidized and reduced forms of glutathione is, however, much more labile, and susceptible to change with the condition of the tissue. Thus the oxidized form increased rapidly on the death of animals, and incubation of the separated tissue in oxygenated glucose media brought about only a partial return to the values *in vivo*. This may at first be thought due to oxygenation during the experiments *in vitro*, for the outer parts of the tissue slice are exposed to higher oxygen tensions than those obtaining *in vivo*. However, even totally anaerobic conditions *in vitro* did not lead to more reduced glutathione. When, during other experiments *in vitro*, the tissue's oxidized glutathione did decrease, the change was very slow. In Fig. 4 it approximated to 0·4 μequiv./g. of tissue/hr., which contrasts with the rate of 500 μequiv./g./hr. at which glutathione reductase can operate in cerebral extracts (McIlwain & Tresize, 1957). If this enzyme is effective in the cell-containing tissue, persistence of oxidized glutathione would presumably reflect a persistence of oxidized 2′-phosphocozymase. In the present simple saline mixtures, substrates are undoubtedly fewer than those supplied by the blood *in vivo*; it is quite feasible that this limitation to the experiments *in vitro* may be reflected in a balance between oxidized and reduced coenzymes in the separated tissue, which differs from that obtaining *in vivo*, and further investigations are in progress.

SUMMARY

1. By a simple modification of the assay of glutathione by yeast apoglyoxalase, both total and reduced glutathione have been determined. Reduction of oxidized glutathione is brought about by the enzyme preparation without additional manipulation.

2. In the cerebral tissues of rats and guinea pigs rapidly fixed *in situ*, total glutathione approximated to 3 equiv. of reduced glutathione/g. and the oxidized form, to 0·05–0·1 μequiv./g.

3. In cerebral tissues removed rapidly at room temperature, from several mammalian species, the content of total glutathione differed little from that quoted, but oxidized glutathione had risen to 0·3–1·5 μequiv./g.

4. During respiration of cerebral tissues *in vitro* their total glutathione could be maintained at concentrations found *in vivo* by incubation in oxygenated glucose media containing added glutathione. If glutathione was not added, lower values were found in the tissue. Oxidized glutathione during experiments *in vitro* was slowly reduced, but after 2 hr. at 37° remained much higher than in the tissue *in vivo*.

We are greatly indebted to Dr L. Christine G. Thomson for carrying out amperometric determinations of glutathione, and to the Nuffield Foundation and to the Board of Governors of the Bethlem Royal Hospital and the Maudsley Hospital for support for these studies.

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