SUMMARY

1. Purified human albumin heated in solution in 0·15 M sodium chloride at pH 6·8 develops a second component.

2. At temperatures of 45° and 50° prolonged heating is required to modify albumin, but at temperatures from 56° to 60° the modification is established in 1 hr.

3. In dilute solution slight but progressive increases in viscosity were noted at 58° if heating was prolonged. Solutions of 5–6·25 % could be heated without gross change in viscosity, but above this protein concentration albumin gels.

4. The action of a number of amino acids and acetyl amino acids on the heat stability of albumin was examined, but all were ineffective except acetyltryptophan, and to a lesser degree acetylphenylalanine.

5. The stabilizing effects of different concentrations of acetyltryptophan were estimated. Solutions containing 20 mg. of acetyltryptophan/g. of albumin may be heated for 10 hr. at 60° without modification of the protein.

6. Moving-boundary electrophoresis proved more sensitive in detecting modification than electrophoresis on paper, the ultracentrifuge, or changes in viscosity.

We would like to thank Dr J. R. Laurence for a sample of 1-anilinonaphthalene-8-sulphonic acid.

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The Speed of Several Cerebral Reactions Involving the Nicotinamide Coenzymes

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A recent survey of respiration in cerebral systems (McIlwain, 1955) showed that although there is qualitative evidence and general expectation that the greater part of cerebral respiration involves nicotinamide coenzymes and cytochromes, known rates of individual reactions oxidizing the reduced coenzymes are too low to account for the oxygen consumption of the brain or of isolated cerebral tissues. Thus respiration of rat or guinea-pig cerebral cortex with glucose and other substrates can reach 120 or 150 μmoles of O_2/g. of tissue/hr.; but the tissue has been observed to reduce by reduced cozymase only 220 μmoles of cytochrome *c*/g./hr., corresponding to 55 μmoles of oxygen/g./hr.

To bridge this gap we have investigated the rates at which cerebral tissues catalyse a number of relevant reactions. These have included oxidation of reduced cozymase by cytochrome *c* and by air, and the corresponding reactions of reduced 2′-phosphocozymase. Interconversion of the two coenzymes has been sought, and also optimum conditions for oxidation of their reduced forms by glutathione.

EXPERIMENTAL

Materials

Cozymase (of 90–95% purity), cytochrome *c* (salt-free preparation of approx. 90% purity) and adenosine triphosphate were from the Sigma Chemical Co. and from Boehringer, Mannheim. Ethylenediaminetetraacetic acid (EDTA) was from Eastman Organic Chemicals.

Reduced cozymase and 2′-phosphocozymase. Specimens of approx. 90% purity were obtained from the Sigma Chemical Co. In addition, solutions of the reduced coenzymes were prepared as follows (see Shuster & Kaplan, 1955; Asnis, 1955). Sodium dithionite (15 mg.) in the stopper of an evacuated Thunberg tube was tipped promptly into 20 mg.

of $NaHCO_3$ and 7 mg. of coenzyme in 2 ml. of water. After 10 min. at 37°, the tube was cooled and opened and O_2 passed vigorously for 5 min. at room temp. The solutions (0·20–0·05 ml. in 3 ml. of water) were assayed absorptiometrically and kept at −20°. For studying glutathione reductase these solutions were not adequate, apparently because they contained materials derived from the dithionite, and the Sigma specimens were used.

In examining the possible interaction of cytochrome *c* and the reduced nucleotides, the following control experiments were carried out. The preparation of reduced nucleotide was incubated with the cytochrome *c* reductase reaction mixture without added tissue, and its absorption at 550 mμ. observed during 10 min. No change was found, nor was change observed in a similar mixture in which the reduced coenzyme solution was replaced by a 'blank' solution made by incubating dithionite and bicarbonate and then oxygenating. Also, the 'blank' solution was incubated in the complete cytochrome reductase mixture with tissue, and no change in absorption observed during 20 min. at 37°. Change at approx. 300 μmoles of cytochrome/g. of tissue/hr. was, however, observed when reduced cozymase was added.

Glutathione. Reduced and oxidized forms were purchased from Light and Co. In addition, the oxidized form was prepared by Rall & Lehninger's (1952) procedure but without attempting to free it from solvent of crystallization; it had an $[\alpha]_D^{12}$ of −97°, suggesting that the specimen retained some 10% of solvent. This was confirmed by its equivalence in oxidation of reduced 2′-phosphocozymase.

Tissue preparations. Organs were removed promptly, weighed and put into ice-cold test-tube homogenizers with 9 vol. of cold water or of 0·25 M sucrose and ground. The suspensions were kept at 0–3° and further dilutions made immediately before use. The part designated brain stem lay ventral to the cerebellum of the guinea pig or rat, and consisted largely of medulla and pons. Cerebral hemispheres consisted of cortical grey and subcortical white matter, without lower structures.

Acetone-dried powders, except in the instance noted below, were made by cooling the tissue in a glass-homogenizer tube, adding 7 vol. of acetone at 0°, and rapidly homogenizing and centrifuging. The supernatant liquid was discarded, the tissue residue re-extracted twice more with acetone as before, and the residue dried *in vacuo*.

In studying the phosphorylation of cozymase the following preparations, based on those of Wang & Kaplan (1954), were employed. In different experiments the brains of guinea pigs and sheep were rapidly ground in 20 vol. of ice-cold acetone, the residue twice further treated with acetone, and dried *in vacuo*. The acetone-dried powders were extracted, in some cases twice, with 11·5 ml. of cold 0·02 M-$KHCO_3$/g. of powder. Protamine sulphate [0·2% in 0·04 M aminotrishydroxymethylmethane (tris), pH 7·5] was added with stirring to the extract (Wang & Kaplan, 1954), precipitates being collected after addition of 8 and 10 ml. of the protamine solution and extracted with 0·2 M acetate buffer, pH 5. Preparations were also made from the $KHCO_3$ extract by precipitating with the 0·2 M acetate buffer.

Methods

A number of preliminary experiments, not described in detail, were performed to ensure that conditions for the estimations were optimum.

Cytochrome c reductase. Conditions initially were based on those of Hogeboom (1949), Mahler, Sarkar, Vernon & Alberty (1952) and Brody, Wang & Bain (1952); the reaction mixture concentrations were 33 mM sodium phosphate, pH 7·4, 0·2 mM-KCN, 35 μM cytochrome *c*, 67 or 100 μM reduced nicotinamide nucleotide and an appropriate tissue preparation. It was found advantageous to adopt Slater's (1950) procedure and mix first the components other than cyanide and reduced coenzyme in spectrophotometer cells in the thermostat, and to leave these together for 10–15 min. for temperature equilibration and for the cytochrome to be completely oxidized by the dissolved O_2 of the solutions.

The reaction was normally followed by a change in absorption at 550 mμ. on reducing the cytochrome. In preliminary experiments the visible spectrum of the reaction mixture was recorded and the bands characteristic of reduced cytochrome *c* were observed to appear. Normally 8–12 readings were taken at intervals of 0·5–1·5 min., according to the speed of the change and the rate calculated from the initial linear group of values; this included at least six readings. In certain cases the change at 340 mμ., due mainly to oxidation of the reduced nucleotide, was followed. Allowance was then made for the concomitant change in cytochrome absorption as described by Hogeboom (1949). Stoicheiometric relationships were demonstrated between the main reactants by adding 4·8 μm-moles of reduced cozymase, which was found to produce a change in extinction at 550 mμ. corresponding to 10 μm-moles of cytochrome *c*.

At 23°, reactants in the concentrations described were demonstrated to be in excess: the initial reaction rate with reduced cozymase at 30 μM was the same as that at 60 μM. With cytochrome *c* at 15 μM it was the same as at 35 μM. Relations at 37° are described below.

Oxidation of reduced nicotinamide nucleotides by air. Reaction mixtures normally contained reagents in the following final concentrations: 33 mM sodium phosphates, pH 7·4, 0·3 mM EDTA, 5 μM cytochrome *c*, 150 μM reduced nicotinamide and a suitable dilution of a tissue preparation. Reagents were added in the order named to sufficient water to give a final volume of 3 ml. in two spectrophotometer cells. A third cell contained the mixture but without nucleotide. Absorption at 340 mμ. in the first two with the third as control was measured during a 10 min. period at 37° to allow temperature equilibration before adding the tissue; it was found to change little if at all during this period. A suitable dilution of a tissue preparation was then added to the first two cells and measurements were made each 0·5–2 min. The solution as ordinarily handled would be expected to be 220 μM with respect to dissolved oxygen from the air, or to contain over four times the amount necessary to oxidize all the reduced nucleotide.

Systems converting cozymase into 2′-phosphocozymase. Evidence for this conversion by cerebral tissues was sought under the conditions in which it had been measured in liver by Wang & Kaplan (1954).

The preparations from brain described above, and also some made from liver by the same procedure, were assayed in a reaction mixture containing reagents in the following concentrations: 6·7 μM cozymase, 8 mM adenosine triphosphate, 10 mM-$MgCl_2$, 100 mM tris, pH 7·5, and 10 mM nicotinamide. In some cases synthesis was examined with one-quarter of these concentrations of reagents, and in

others nicotinamide was omitted. Reaction mixtures (0·5 ml.) were incubated for times between 10 and 120 min. and deproteinized by heating. 2′-Phosphocozymase was then determined with *iso*citric dehydrogenase of pig heart by the method of Ochoa & Weisz-Tabori (1948) as modified by McIlwain & Rodnight (1949). It was confirmed that liver extracts were capable of the conversion, rates of 0·5 μmole of 2′-phosphocozymase formed/g. of tissue/hr. being observed. Small quantities (0·1 μmole) of 2′-phosphocozymase added to the cerebral reaction mixtures were recovered.

Glutathione reductase. Initial conditions were based on those of Rall & Lehninger (1952) and Asnis (1955); the concentrations of reagents in the reaction mixture were 50 mM sodium and potassium phosphates, pH 7, 0·5 mM oxidized glutathione, 36 μM reduced 2′-phosphocozymase and an appropriate tissue preparation. These were later modified as described in the Results section.

Absorptiometric measurements. These were made with a Hilger Uvispek mark II photoelectric spectrophotometer. The cell compartment was fitted with a thermometer and a water jacket through which water from a thermostatically controlled bath was circulated. Quartz cells of 1 cm. light path were employed, some taking 3 ml., and others 0·23 ml., of fluid. The change in extinction coefficient at 550 mμ. on reducing cytochrome *c* was taken from the data of Haas, Horecker & Hogness (1940) and of Slater (1950) as 18·5 cm.²/μmole. The change at 340 mμ. on oxidizing reduced cozymase and its 2′-phosphate was taken as 6·22 cm.²/μmole (Horecker & Kornberg, 1953).

RESULTS

Cytochrome c *reductases*

Reduction of cytochrome *c* by reduced cozymase was initially measured under conditions described by previous investigators (see above) and proceeded in suspensions from guinea-pig brain at 100–300 μmoles/g./hr. (Fig. 1; Table 1). Rates in different experiments varied considerably and fell if the tissue was left for a few minutes at 37° before reaction was commenced by adding reduced cozymase. Added substances were included in the reaction mixture as possible activating or stabilizing agents. Glutathione, which could itself reduce cyctochrome *c* in this reaction mixture, did not lead to higher rates, nor did glycylglycine; but serum albumin, and especially EDTA, did so. This acid has been used by Bonner (1954) in the succinic oxidase system of heart. Rates between 400 and 1000 μmoles of cytochrome *c* reduced/g. of tissue/hr. were then obtained for different parts of the brain, and with liver some 2500 μmoles were reduced/g./hr.

For maximum rates it was found necessary to prepare the tissue suspension in sucrose, and to carry out any dilution in sucrose just before the assay. With sucrose suspensions in reaction mixtures containing EDTA, areas of the brain stem proved to be of high and reproducible activity in reducing cytochrome *c* by reduced cozymase, and with such preparations conditions for optimum activity were further explored. The activity was not significantly altered when the EDTA concentration was varied between 0·13 and 1·33 mM, and a concentration of 0·33 mM was adopted. When phosphates were used as the main inorganic component in reaction mixtures, highest rates were obtained with solu-

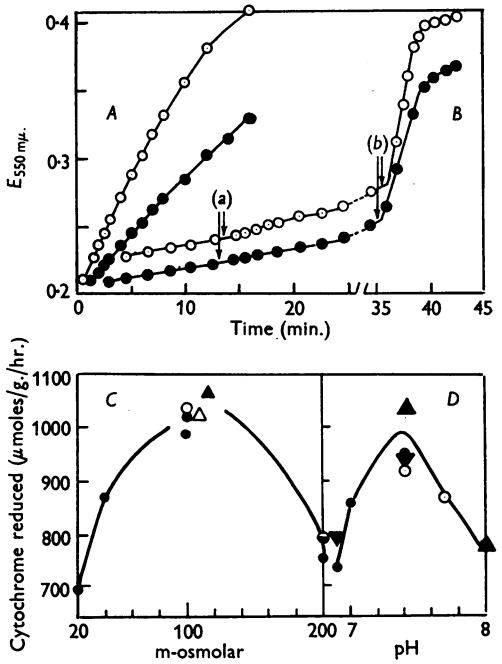

Fig. 1. Reduction of cytochrome *c* by reduced nicotinamide coenzymes. *A*, Change measured in the reaction mixture of Table 1, but with 0·3 mM EDTA and 0·25 mg. (○) and 0·125 mg. (●) of guinea-pig midbrain/3 ml. *B*, Change measured in the reaction mixture of Table 1, with (○) and without (●) mM EDTA and with 0·625 mg. of guinea-pig midbrain and 10 μmoles of reduced 2′-phosphocozymase present initially/3 ml. A further 50 μm-moles of the phosphocozymase were added at (*a*) and 50 μm-moles of reduced cozymase at (*b*). *C*, Rate of reduction of cytochrome by reduced cozymase in the reaction mixture of Table 1 containing 0·3 mM EDTA, but in which the inorganic constituents were altered as follows: ●, Phosphate molarity altered as indicated on the abscissa; ○, phosphate replaced by NaCl; ◒, equimolar phosphate and glycylglycine; △, 13 mM phosphate and 33 mM-NaCl; ▲, 5·5 mM phosphate and 50 mM-NaCl. In expressing concentrations in osmolar units the phosphates were assumed to yield 3 equiv./mole and the NaCl 2 equiv. Rates were derived from experiments such as that of Fig. 1*A* with guinea-pig midbrain. *D*, Effect of phosphate buffers (33 mM) of different pH on the reduction in the reaction mixture of Table 1, by reduced cozymase. The differently shaped points refer to different preparations of guinea-pig brain stem.

Table 1. *Cytochrome c reductase of guinea-pig tissues*

Reaction mixtures contained 33 mM sodium phosphate, pH 7·4, 35 μM cytochrome *c*, 0·2 mM-KCN, 0·1 mM reduced coenzymes and 0·1–0·2 mg. of tissue/ml. Rates quoted are the mean of two values agreeing to within 5%, except when followed by standard deviation and, in parenthesis, the number of experiments. When two values are quoted they refer to different animals. Rates with glutathione were corrected for rate of reduction in the absence of reduced cozymase.

Tissue	Suspension fluid	Additions to reaction mixture	Rate of cytochrome *c* reduction (μmoles/g./hr.) By reduced cozymase	By reduced 2′-phospho-cozymase
Whole brain	Water	None	115*, 120*	—
		None	272±11 (4)	15
Brain stem	0·25 M Sucrose	None	470±16 (6)	20
		EDTA, 0·33 mM	883±24 (6)	12, 16
		Plasma albumin, 1%	560	—
		Glutathione, 2 mM	463	—
Liver	Water	None	730, 750	—
	0·25 M Sucrose	EDTA, 0·33 mM	2460, 2540	—

* At 22°.

tions about 30 mM in phosphate, which is the concentration recommended by previous workers but is markedly hypotonic (Fig. 1*C*). It could be replaced by NaCl of similar tonicity (50 mM), and a little phosphate added to the NaCl had little effect on the rate (no attempt has been made to render the tissue preparation deficient in phosphate). A pH of 7·4 proved to be optimum (Fig. 1*D*), again in agreement with findings with other tissues. The concentration of reduced cozymase in the normal reaction mixture (67–100 μM) appeared to give optimum rate, as indicated by the linear course of the greater part of the reaction curves of Fig. 1*A*. It was, however, important to investigate this directly, and results are given in Fig. 2*A*. Here the rate is seen to reach its maximum at about 100 μM cozymase; half-maximal rate was at some 20 μM.

Each part of the brain examined was also capable of slowly reducing cytochrome *c* in presence of 2′-phosphocozymase. The rate (Table 1) averaged about 5% of that with cozymase, when the reduced phosphocozymase was examined at 0·02–0·1 mM. The presence of 0·06 mM reduced phosphocozymase, did not affect the rate of reduction of cytochrome *c* by reduced cozymase added later in the same concentration although a change in rate of 10% would have been detectable. No information has been obtained which might indicate whether a single system oxidizes the two coenzymes.

Nicotinamide nucleotide oxidases

Reduced cozymase was relatively slowly oxidized when added to ground cerebral tissues in salt mixtures (Table 2). Rates were found of some 30 μmoles oxidized/g. of brain stem/hr. in comparison with an observed respiratory rate of up to 100 μmoles of O_2 absorbed/g./hr., suggesting the simple conditions to be inadequate. Findings just

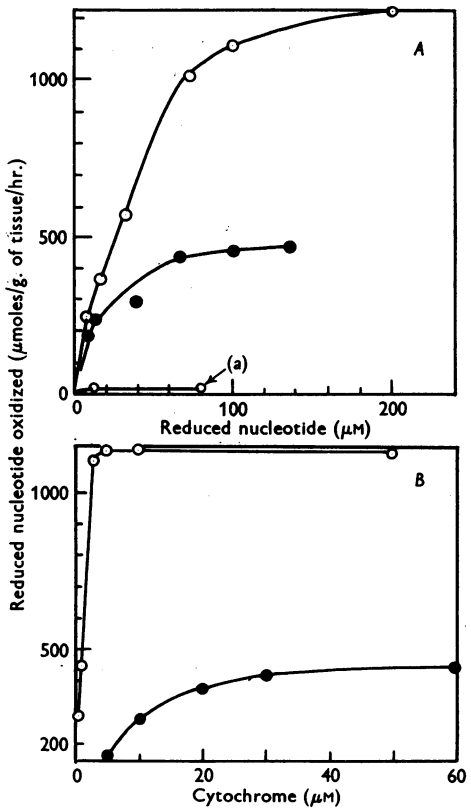

Fig. 2. Rate of oxidation of reduced nicotinamide nucleotides (cozymase except at *a*, when phosphocozymase was used) by air (○) and by cytochrome *c* (●). *A*, Dependence of the rates on the concentration of reduced nucleotides. *B*, Dependence on cytochrome *c*. Reaction mixtures for reduction of cytochrome *c* by reduced nucleotides as in Fig. 1*A* except where indicated; for oxidation of reduced cozymase, the full reaction mixture of Table 2.

Table 2. *Requirements for oxidation of reduced cozymase with oxygen by cerebral suspensions*

The complete reaction mixture contained 33 mM phosphate, pH 7·4, 33 μM EDTA, 5 μM cytochrome *c* and 150 μM reduced cozymase. Tissues were ground in 0·25 M sucrose and used at 0·1–0·2 mg./ml. Mean, standard deviation and number of experiments are given in three cases; other values are averages of duplicates agreeing to within 10%.

Tissue	Reaction mixture	Loss of reduced cozymase (μmoles/g./hr.)
Brain stem, guinea pig	Complete	781±27 (6)
	No cytochrome	114
	No cytochrome, no EDTA	29
	No cytochrome, no EDTA; with 10 mM histidine	115
	No EDTA	400
	No EDTA; with 10 mM histidine	730
Brain stem, rat	Complete	1056±34 (6)
	With 0·1 or mM adenosine diphosphate	1010
	No cytochrome	92
	No cytochrome; with 0·3 mM adenosine diphosphate	108
	No EDTA	270
	No EDTA; with 0·3 mM adenosine diphosphate	290
Cerebral cortex, rat	Complete	616±28 (4)

described in relation to cytochrome reductases, and also those of Slater (1950) and Bonner (1954) on oxidation of reduced cozymase by other tissues, prompted the addition of cytochrome *c*, adenosine diphosphate, inorganic phosphate, histidine and EDTA. With certain of these additions, rates were increased 20- to 40-fold.

Two main requirements were found: for cytochrome *c*, and for either histidine or EDTA (Table 2). Relatively little EDTA was found necessary; rates with 330 and 33 μM were similar and the latter concentration was adopted for further work. It could be replaced by 10 mM histidine. Requirements for cytochrome *c* by the oxidase system were much lower than those of cytochrome reductase; 5 μM gave the same rate as 50 μM and half-maximal rate was given at about 0·6 μM concentration of cytochrome *c* (Fig. 2*B*). This concentration, although low, corresponds to a turnover rate of only some 200 moles of cozymase formed/mole of cytochrome/hr. Values of similar magnitude can be derived from Slater's (1950) data for oxidation of reduced cozymase by kidney preparations.

The reaction proved to be maximal at about pH 7·4 (Fig. 3). It showed a requirement for inorganic salts which was met by NaCl or K_2SO_4 (Table 3), which afforded higher reaction rates than did glycylglycine. Beyond this general requirement two ions were found to have specific effects: phosphate in accelerating the reaction and calcium salts in depressing it. The lower rate with 1·4 mM-$CaCl_2$ was similar to that found in the absence of EDTA. The presence of lower concentrations of calcium salts, 0·08 and 0·24 mM, which were below that of the EDTA (0·3 mM, equivalent to 0·6 mM Ca^{2+}), had little effect on the reaction.

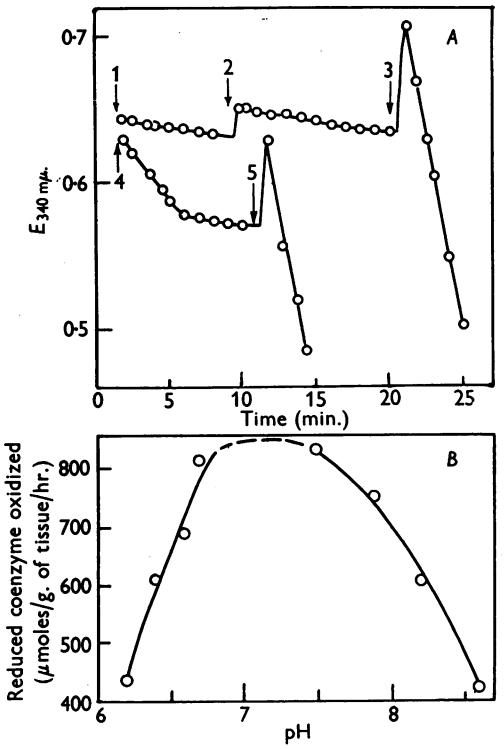

Fig. 3. Oxidation of reduced nicotinamide nucleotides with oxygen by suspensions of brain stem. *A*, Rat tissue, 0·33 mg./ml. with full reaction mixture of Table 2 except for nucleotides, which were added as follows: at 1, 16·7 μM, and at 2, 100 μM phosphocozymase; at 3 and 5, 100 μM, and at 4, 16·7 μM reduced cozymase. *B*, Full reaction mixture of Table 2 with 33 mM phosphate buffers of the pH indicated; guinea-pig medulla ground in sucrose was added at 0·33 mg./ml.

Table 3. *Oxidation of reduced cozymase: effect of inorganic salts*

Experiments were with rat brain stem suspended in sucrose and (except when otherwise indicated) added to the full reaction mixture of Table 2, with 0·3 mM EDTA. Concentrations of constituents listed were chosen to give 96–106 osmolar solutions, assuming the phosphate and K_2SO_4 to yield 3 equiv. and KCl 2 equiv.

Constituents of media (mM)			Rate of oxidation (μmoles of cozymase/g./hr.)
Phosphates	NaCl	Others	
33	0	0	1090
10	33	0	1130
25	13	0	1060
0	50	0	805
0	13	Glycylglycine, 80	565
0	0	Glycylglycine, 100	450
0	0	K_2SO_4, 33	815
30	0	KCl, 2·7; $MgSO_4$, 0·7	990
30	0	KCl, 2·7; $MgSO_4$, 0·7; $CaCl_2$, 1·4	560
30	0	KCl, 2·7; $MgSO_4$, 0·7; $CaCl_2$, 0·08 or 0·24	1030

Cozymase and phosphocozymase. The reaction mixture based on the findings of the preceding paragraphs contained cytochrome *c* and EDTA and was buffered with phosphates at pH 7·4. In it, appreciable concentrations of reduced cozymase were found to be needed for optimum reaction rate (Fig. 2*A*), half-maximal oxidation being reached with about 40 μM reduced cozymase. Reaction with reduced 2′-phosphocozymase was much slower, averaging with brain stem 20–30 μmoles/g./hr. (Fig. 2). This was obtained with low concentrations of phosphocozymase, of no more than 10 μM. Reduced cozymase, added to suspensions subsequently to 20 or 80 μM reduced phosphocozymase, was oxidized at normal rate.

Glutathione reductase

Oxidation of reduced 2′-phosphocozymase by cerebral as by other tissues has been found to be greatly accelerated by addition of oxidized glutathione, which underwent concomitant reduction (Fig. 4; see Vennesland & Conn, 1954). Conditions for assay of the reductase were developed from those of Rall & Lehninger (1952), but the preparation of the tissue and the reaction mixture were found to need more detailed examination.

Extraction. Though liver ground in sucrose was described as of the same reductase activity as an extract of an acetone-dried powder of the tissue (Rall & Lehninger, 1952) this was not found to be the case with cerebral tissues. Preparations in which the tissue was ground in sucrose were found of lower activity than others ground in water, and these of lower activity than extracts of acetone-dried powder. Other manipulations were therefore examined in searching for conditions in which the tissue exhibited maximal activity (Fig. 4*B*). Keeping the tissue suspension in 9 vol. of water at 2–3° led to a slow increase in activity; greater

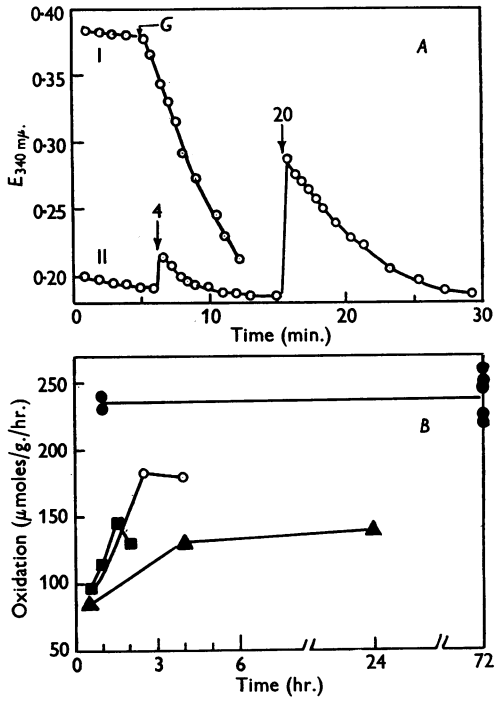

Fig. 4. Glutathione reduction by guinea-pig cerebral tissue with reduced 2′-phosphocozymase in the full reaction mixture quoted on p. 294, except as specified. *A*, Phosphate extract of acetone-dried cerebral hemisphere with: I, 83 μM reduced nucleotide present initially and 300 μM glutathione added at *G* (0·3 subtracted from all ordinates); II, with initially 300 μM glutathione and no added nucleotide; concentrations indicated (μM) were added as shown. *B*, Rate of oxidation of the reduced nucleotide by different preparations (see text) of guinea-pig cerebral hemispheres. ●, Acetone-dried material, extracted with phosphate, pH 7, and the extract kept at 2° for the time indicated. Tissue ground in water, and then kept: ▲, at 2° alone; ○, at 2° with *iso*butanol; ■, at 37° alone.

increase was obtained on keeping at 37° and again greater at 2–3° with *iso*butanol. These levels remained below that of acetone-dried powder extracted with either water or phosphate (50 mM, pH 7). Preparations in aqueous ethanol also gave lower rates. Activities both of the acetone-dried powders and of extracts from them were stable for several days (probably for longer) and were therefore accepted as a basis for assay.

Reaction mixture and conditions of assay. In unfractionated extracts of guinea-pig cerebral hemispheres, glutathione reductase has not proved exacting in its requirement for inorganic salts. The 50 mM sodium phosphates used previously could be largely replaced by mixtures of glycylglycine and NaCl (Table 4); replacement of the NaCl by Na_2SO_4, or its omission, gave lower rates. EDTA increased rates by about 60 % and was included in the standard reaction mixture. The following materials had little effect on reaction rate: 0·3 mM cysteine, 0·1 mM $MnCl_2$ and a crude specimen of flavine–adenine dinucleotide. The reaction was 25 % faster at pH 7 than at pH 8, and 15 % faster at pH 7 than at pH 6.

On this basis the concentrations of reagents in the full reaction mixture used in the following experiments were 50 mM sodium and potassium phosphates at pH 7, 330 μM EDTA, 83 μM reduced 2′-phosphocozymase, 300 μM oxidized glutathione and a preparation equivalent to 0·1–5 mg. of tissue/ml.

With the normal excess of oxidized glutathione, added limited quantities of reduced coenzyme were found to be almost quantitatively removed (Fig. 4*A*). With excess of reduced coenzyme, limited quantities of oxidized glutathione gave a change in extinction showing that almost 1 equiv. of reduced coenzyme had been removed, actual quantities being: added, 45 μmoles; removed, 41, 45 μmoles. When in this mixture the concentration of reduced phosphocozymase was decreased, marked dependence of reaction rate on the coenzyme was observed at levels below 20 μM (Fig. 5). When the oxidized glutathione was varied in level, concentrations below 250 μM were associated with lower rates (Fig. 5). The average rate observed with preparations from guinea-pig cerebral hemispheres

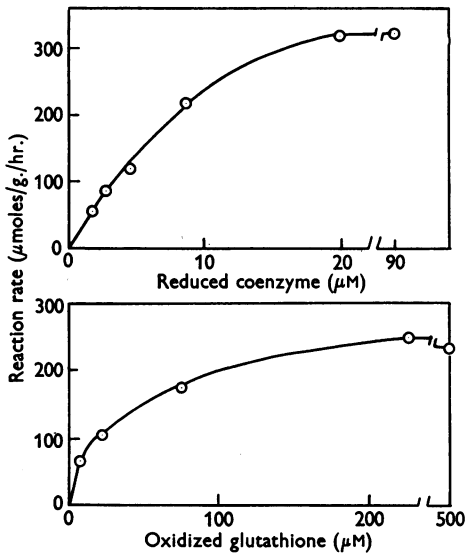

Fig. 5. Glutathione reductase of guinea-pig cerebral hemispheres: dependence of rate on concentration of reactants. Reaction mixture contained acetone-dried preparations in the reaction mixture of this page, with 83 μM reduced 2′-phosphocozymase and 300 μM oxidized glutathione except when these were altered as indicated.

Table 4. *Glutathione reductase of guinea-pig cerebral tissues and of liver*

Results are expressed as in Table 2. Full reaction mixture is described in the text above.

Preparation	Departure from full reaction mixture (mM)	Rate (μmoles of reduced 2′-phosphocozymase lost/hr./g. of tissue)
Cerebral hemisphere		
A. Phosphate extract of acetone-dried powder	None	240±9 (5)
	No EDTA	150
	Phosphates, 0·4; glycylglycine, 100	175*, 190
	Phosphates, 0·4; glycylglycine, 33; NaCl, 33	250*
	Phosphates, 0·4; glycylglycine, 33; K_2SO_4, 22	250*
B. Ground in water; used immediately	None	98
	No EDTA	56
C. Preparation *B* after keeping at 2° for 24 hr.	None	140
	$MgSO_4$, 0·2; KH_2PO_4, 0·2; KCl, 1; NaCl, 21	105
Liver	None	980

* The further addition of 3·3 mM sodium phosphate, pH 7, made no change in these rates.

in the full reaction mixture was 250 μmoles of coenzyme reacting/g. of fresh tissue/hr., or some six times that previously reported in rat brain (Rall & Lehninger, 1952).

Other reactions

Conversion of cozymase into 2′-phosphocozymase has been sought, as described in the experimental part, but the rate of any conversion appeared to be less than 0·1 μmole/g./hr. Under the conditions in which reduced 2′-phosphocozymase was oxidized by cytochrome *c* or by air, any rapid dephosphorylation would have been shown, but was not detected; whether the slow oxidation occurring was an immediate oxidation of the 2′-phospho compound or was secondary to other changes, has not been determined.

DISCUSSION

Metabolic routes concerning cozymase and 2′-phosphocozymase in cerebral tissues are thus relatively distinct. Interconversion of the coenzymes by phosphorylation of cozymase was much slower than in liver; the main oxidation of reduced cozymase through cytochromes proceeded much more rapidly than did the corresponding reaction in phosphocozymase; and an oxidation corresponding to that of reduced phosphocozymase by glutathione scarcely proceeded with reduced cozymase.

Cozymase. The previous discrepancy (see Introduction) between rates of respiration in cerebral tissues and their rates of oxidation of reduced cozymase has received its explanation largely in terms of methods of measuring the oxidation, rather than in terms of alternative reactions in the coenzymes. Reduced cozymase was oxidized in a mixture containing inorganic phosphate, added cytochrome *c*, and EDTA or histidine, at some 600 μmoles/g. of cerebral cortex/hr. This, equivalent to 300 μmoles of oxygen/g./hr., is approximately twice the greatest rate of absorption of oxygen which has been observed in cerebral systems *in vivo* or *in vitro* (see McIlwain, 1955). The rate of 600 μmoles/g./hr. was reached in tissue suspensions at pH values and at salt levels corresponding to those of the intact tissue. It may, however, be limited in the intact tissue by the available reduced cozymase. Although the total cozymase of rat brain is about 300 μm-moles/g., on the average more than one-half of this is oxidized, leaving some 130 $\pm$ 60 μm-moles/g. in the reduced form (Glock & McLean, 1955). Thus (see Fig. 2*A*), in some specimens the concentration of reduced cozymase may be adequate for only one-half of the maximal rate of oxidation. This computation and also later ones do not make allowance for the extracellular volume of the tissue, nor for any binding of cozymase, factors which would affect the available concentration of coenzyme in opposing senses.

Estimation of the rate of oxidation of reduced cozymase by cytochrome *c* in the mixtures containing cyanide appears to give a measure of only part of the potential activity of cytochrome reductase. From the rate of the oxidation of reduced cozymase by oxygen, accelerated by cytochrome *c* (600 μmoles of coenzyme/g./hr.), reduction of cytochrome *c* at 1200 μmoles/g./hr. must be involved. Rates actually observed, though higher than those of Brody *et al.* (1952) (220 μmoles/g./hr.), were, in cerebral cortex, about 400 μmoles/g./hr. That this is lower than 1200 is presumably due to difficulty of access of a succession of cytochrome *c* molecules to the enzyme during their reduction; a succession which may not be necessary when the cytochrome is an intermediary carrier rather than a major reactant. The concentration of cytochrome *c* found necessary in the oxidation by air was 3 μM (Fig. 2) in distinction to some 30 μM for the lower maximal rate reached in the reductase system. Previous observations supporting relatively high values for the native cytochrome reductase can be deduced from data of Wang & Bain (1953) and of Abood, Gerard, Banks & Tschirgi (1952), who found for reactions in rat brain in which cytochrome reductase was probably involved, rates corresponding to about 800 μmoles of coenzyme oxidized/g./hr. and 400 μmoles/g./hr. respectively.

2′-Phosphocozymase. Oxidation of the reduced phosphocozymase by cytochrome in the mixture containing cyanide reached some 10 μmoles of coenzyme/g. of tissue/hr., again about one-half of that of its oxidation by oxygen. Such oxidation could not account for more than 15 % of the tissue's maximal oxygen uptake. The quantity of 2′-phosphocozymase in rat brain is also relatively small (Gore, Ibbott & McIlwain, 1950): some 12 μm-moles/g., almost all in the reduced form (Glock & McLean, 1955). Related to this are two aspects of the present findings: first, in a 12 μM solution, reduced phosphocozymase is already being oxidized by oxygen at the maximal rate (Fig. 2*B*). Further, the rate of oxidation of reduced phosphocozymase by oxygen is some 2–5 % of the rate of oxidation of reduced cozymase, just as the quantity of phosphocozymase in the tissue is some 2–5 % of the quantity of cozymase.

The present search for optimum conditions for displaying the glutathione reductase of cerebral tissues has emphasized that this system forms the major route for oxidation of reduced 2′-phosphocozymase. Rates now observed reach 250–300 μmoles of glutathione or coenzyme reacting/g. fresh wt. of tissue/hr., or six times those earlier recorded. They thus correspond to 125–150 μmoles of oxygen/g./hr., which is greater than the normal rate of

respiration of the brain *in vivo* or *in vitro*, and falls not far short of maximal stimulated rates. Moreover, rates approaching the maximal can be reached with levels of reduced coenzyme and oxidized glutathione which exist in the brain. Considering the reduced coenzyme, the native level of 12 ± 4 μm-moles/g., if regarded as equivalent to a 12 ± 4 μM solution, could support oxidation at 180–300 μmoles/g./hr. (Fig. 5). Normally, however, this reaction is probably limited by the concentration of oxidized glutathione in the brain, which may be very low as it is in several organs of the body (Bhattacharya, Robson & Stewart, 1955). Fujita & Numata (1938) found the total glutathione in rat brain to be 2·7 μmoles/g., without detectable oxidized form, probably implying this to be less than 0·05 μmole/g. Kudryavtseva & Kudryavtseva (1950), however, suggest that up to one-fifth of the cerebral glutathione may be in the oxidized form in the brain of young rabbits. As the present findings indicate about 80 μM oxidized glutathione to be needed for half-maximal rate of the reductase, the different estimates give very different impressions of the rate at which the reductase is likely to proceed *in vivo*, and the levels are being re-examined.

Rates reported for systems reducing 2′-phosphocozymase in rat brain are: glucose 6-phosphate dehydrogenase, 20 μmoles/g./hr.; breakdown of ribose 5-phosphate, 45 μmoles/g./hr. (Glock & McLean, 1954). Successive operation of the systems could thus produce reduced phosphocozymase at 40 μmoles/g./hr. Regarding the remainder of a maximal respiratory rate of 150 μmoles of oxygen/g./hr. (i.e. 130 μmoles/g./hr.) as proceeding through the tricarboxylic cycle, when one-sixth of the oxidation is via *iso*citric dehydrogenase which reduces phosphocozymase, a further 43 μmoles/g./hr. of the reduced coenzyme could be formed, giving a total of 83 μmoles/g./hr. Systems now observed to remove the reduced coenzyme operate maximally at over three times this rate ($250 + 20$ μmoles/g./hr.). Thus the preponderance of reduced forms of the coenzyme and of glutathione in the tissue implies relatively slow oxidation of reduced glutathione. Rates of reactions oxidizing reduced cozymase can similarly be computed to be greater than those which form it. In accordance with this, the tissue's cozymase is found to be predominantly in the oxidized form.

SUMMARY

1. Optimum conditions for determining cytochrome *c* reductase in cerebral tissues involved their grinding in sucrose, the inclusion of ethylenediaminetetraacetate, of reduced cozymase at or above 100 μM and of cytochrome *c* at or above 30 μM. Up to 900 μmoles of cytochrome could then be reduced/g. of tissue/hr.

2. Oxidation of reduced cozymase by air also required ethylenediaminetetraacetate or a surrogate, reduced cozymase at or above 100 μM and added cytochrome *c* at or above 5 μM. It then proceeded at up to 1050 μmoles/g./hr.

3. Reduced 2′-phosphocozymase was oxidized only slowly in the preceding mixtures, but much more rapidly with added oxidized glutathione, in the presence of extracts of acetone-dried tissue, when rates up to 250 μmoles/g. of tissue/hr. were observed.

4. Processes interconverting the two coenzymes or their two reduced forms were relatively slow.

5. The rates of oxidation quoted are many times those previously recorded and are collectively much greater than those of the oxygen uptake of intact cerebral tissues *in vivo* or *in vitro*. Concentrations of the reduced coenzymes and of glutathione which occur in cerebral tissues approach but are not usually in excess of the minimal needed for maximal oxidation.

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