assume that other forms of electronic energy, not connected with the radiative transition but with other degrees of freedom, might easily be absorbed by purines. This would lead to the existence, in certain degrees of freedom of the purine molecule, of an energy content in excess of that which could be accounted for by a random distribution and the principle of the equipartition of energy. The presence of adenine in several prosthetic groups and coenzymes, where the purine seems to play no part in the catalysed reaction, is an indication that some general property of this type of compound, such as the one suggested, may be involved.

SUMMARY

1. A study of the fluorescence of riboflavin and of flavin-adeninedinucleotide, and the effect of different quenchers on the former has been made.

2. The quenching of the fluorescence of riboflavin by purines is due to the formation of a complex

having a heat of dissociation of 1.6 ± 0.6 kg.cal./mol. and a lifetime longer than 10^{-6} sec.

3. The small fluorescent efficiency of flavin-adeninedinucleotide in comparison with that of riboflavin is due to the formation of an internal complex between the *isoalloxazine* and the adenine of the molecule. The complex is non-fluorescent and exists in equilibrium with its fluorescent form, the equilibrium being easily displaced by changes in pH.

4. A study of the quenching of the fluorescence of riboflavin by acids and alkalis, shows that the fluorimetric method cannot be used for the determination of the dissociation constant of the anionic form of riboflavin, but its use is justified in the case of the cationic form.

5. A complex of riboflavin with silver ions is described.

6. The variations in the fluorescence of flavo-proteins is discussed, and a relation between the quenching properties of adenine and its presence in prosthetic groups and in coenzymes is suggested.

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The Cozymase of Mammalian Brain

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The cozymase of brain has been studied because of the central importance of this compound in carbohydrate metabolism, and also because in brain as a whole cozymase exists together with very active systems which degrade it. Inhibition of the degradation appears to be accompanied by disturbance of the balance of carbohydrate metabolism (McIlwain & Grinyer, 1950). The present paper describes studies of the normal cozymase content of mammalian brain, of the stability of cozymase in the tissue during various manipulations, and of its possible origin.

EXPERIMENTAL

Determination of cozymase

Apozymase system

Preparation of apozymase from yeast, and its use for determining cozymase, were based on the descriptions of Myrbäck (1933), Euler (1936), Axelrod & Elvehjem (1939) and McIlwain & Hughes (1948).

Apozymase. Fresh bottom yeast obtained from Barclay Perkins and Co. Ltd., to whom we are indebted, was sucked as dry as possible on a Büchner filter, pressed through a wire sieve (0.1 in. mesh) and dried overnight by a fan. The

dried yeast was stored over CaCl_2 at 4° ; its activity declined after 2-3 months' storage. For the preparation of apozymase, it was washed and dried as described by Axelrod & Elvehjem (1939). With some batches of dried yeast, two washings between each drying were sufficient to remove the cozymase and to yield preparations with high fermentation rates when cozymase was added, but with low rates without cozymase. The apozymase was prepared weekly and stored in a refrigerator over CaCl_2 . The rate of fermentation of glucose by apozymase preparations declined after about 10 days' storage.

Hexose diphosphate. Hexose diphosphate was obtained commercially as the Ba salt, and one batch was prepared by the method of Neuberg & Lustig (1942). Commercial samples were fractionally precipitated by ethanol at pH 4.0 (Robison & Morgan, 1930), to reduce their content of inorganic phosphate. A solution of Na hexose diphosphate was prepared each day. The Ba salt was dissolved in ice-cold N-HCl , $2\text{M-Na}_2\text{SO}_4$ added in slight excess to remove the Ba, the solution brought to pH 6.3 with N-NaOH , diluted with water so that the organic P was 10 mg./ml., and centrifuged.

Cozymase estimation was carried out at 30° with Warburg manometers and conical single side-armed flasks. The main compartment contained hexose diphosphate, glucose and Mg and Mn salts in the concentrations recommended by Axelrod & Elvehjem (1939). The final phosphate concentration of the reaction mixture was 0.065 M, which was the optimum concentration for the strain of yeast used. The solution whose cozymase was to be determined was placed in the side arm and the final volume brought to 2 ml. with water. Apozymase (0.2 g.) was added in powder form to each flask immediately before incubation commenced.

After equilibration at 30° for 9 min. the flasks were tipped and readings taken at 3 min. intervals for 21 min. and then at 5 min. intervals. After a short induction period, the CO_2 evolution in each vessel reached a steady rate which was maintained for 20-25 min. The rate of evolution of CO_2 was proportional to the amount of cozymase added to the reaction mixture up to quantities of 15 or 20 μg . pure cozymase, but it was found convenient to make most of the estimations in the range 1-5 μg . cozymase.

A control flask without added cozymase and one containing standard cozymase were included in each set of estimations. A specimen of cozymase obtained commercially and found to be 53% pure was usually employed as standard, and results were calculated in terms of pure cozymase. The cozymase content of the standard specimen was determined as described by McIlwain & Rodnight (1949)

and was found to agree approximately with the value obtained with Slater's (1950) method, for details of which we are indebted to Mr C. Kratzing and Dr E. C. Slater. The activity of the enzyme system (rate of evolution of $\text{CO}_2/\mu\text{mol}$. cozymase) varied with different apozymase preparations, which were therefore not suitable (as implied by McIlwain & Hughes, 1948) for assessing the purity of a cozymase specimen without the use of a standard specimen of known purity. Values ranged from 3 to 8 μmol . $\text{CO}_2/\mu\text{g}$. cozymase/hr. with an average of 5 μmol . $\text{CO}_2/\mu\text{g}$. cozymase/hr. The system was found to estimate dihydrocozymase as cozymase. Adenosinetriphosphate, nicotinamide and nicotinic acid did not affect the determinations.

Extraction of cozymase

The cozymase content of tissue extracts and fresh tissues has been studied in the present investigation. Phosphate buffer (0.1 M, pH 6.2) has been used as the basis of the extracting fluid because it formed part of the apozymase system which was used for the estimations of cozymase, and also because pH 6.2 is close to the pH of optimum stability of cozymase. Heating in this buffer alone for 15 min. at 70° was found to cause little destruction of cozymase. Good recoveries of cozymase added to saline extracts of fresh brain homogenates, or to acetone-dried brain, were obtained after this treatment (Table 1).

This procedure, however, was found to be quite inadequate for the extraction of cozymase from fresh tissue. It was suspected that the loss might be due in part to the action of the enzyme degrading cozymase; nicotinamide which inhibits the action of this enzyme was therefore added to the buffer solution used for extraction. Nevertheless, only 6% of the cozymase added to a brain homogenate was recovered, after heating for 15 min. at 70° in phosphate buffer at pH 6.2 which contained 0.1 M-nicotinamide (Table 1). It was found, however, that by heating for only 2 min. at 100° 90-94% recovery of added cozymase could be obtained. The presence of the nicotinamide was important: it considerably improved the yield of cozymase from fresh tissue (Table 2). For extraction of fresh tissue and tissue slices, a small piece of weighed tissue was dropped into the extracting fluid already at 100° , broken with a glass rod, and kept at 100° for 2 min. Tissue homogenates were pipetted directly into the extracting fluid at 100° .

The efficiency of the methods of extraction has been judged also by comparing the yields of cozymase from fresh tissue, with their content of nicotinic acid after hydrolysis (see below). In computing cozymase content, no correction has been made for the possible loss of some 8% during extraction.

Table 1. *Recovery of cozymase added to brain preparations*

(The extracting fluids consisted of a 0.1 M solution of nicotinamide in phosphate buffer, pH 6.2, of a molarity which gave a final phosphate concentration of 0.1 M. To this buffer, in small tubes in a bath at the temperature of extraction, were added the tissue preparations with or without additional cozymase.)

Preparation	Extraction	Cozymase			
		In tissue (μg .)	Added (μg .)	Recovered (μg .)	Recovery (%)
Saline extract of fresh tissue	15 min. at 70°	0	2.35	2.30	98
Saline extract of acetone-dried tissue	15 min. at 70°	0	3.75	3.75	100
Tissue homogenate	15 min. at 70°	0	14.7	0.9	6
Tissue homogenate	2 min. at 100°	1.93	3.0	4.76	94
Tissue slice	2 min. at 100°	2.0	2.5	4.35	93

Determination of nicotinic acid

Determinations of the total nicotinic acid content of guinea pig cerebral cortex were made on homogenates after hydrolysis of the tissue for 1 hr. at 100° in 2*N*-HCl. The supernatant solution after hydrolysis was decolorized by zinc hydroxide precipitation according to Friedemann & Barborka (1941) and nicotinic acid determined as described by McIlwain & Rodnight (1949).

RESULTS

Cozymase content of different tissue preparations

Cozymase and nicotinic acid. The cozymase content of different preparations of guinea pig brain is given in Table 2. Values for fresh brain were obtained by killing the animal by a blow on the neck, bleeding and removing the brain immediately. A

Table 2. *Cozymase content of brain preparations*

(Extraction conditions, except where otherwise indicated, as described in Table 1, heating 2 min. at 100°.)

Brain preparation	Extraction	Cozymase found		
		No. of samples	Mean (μg./g. fresh tissue)	Standard deviation (μg./g. fresh tissue)
Guinea pig cerebral hemisphere	0.1 <i>M</i> -phosphate with nicotinamide	7	159	11.7
Rat cerebral hemisphere	0.1 <i>M</i> -phosphate with nicotinamide	12	158	16.3
Mouse cerebral hemisphere	0.1 <i>M</i> -phosphate with nicotinamide	4	333	25.8
Rabbit cerebral hemisphere	0.1 <i>M</i> -phosphate with nicotinamide	—	103	—
Guinea pig cortex slices, fresh	0.1 <i>M</i> -phosphate with nicotinamide	7	152	16.3
Guinea pig cortex slices, suspended in Krebs-Ringer solution for 5 min.	0.1 <i>M</i> -phosphate with nicotinamide	20	109	16.4
Guinea pig cortex slices, fresh	0.1 <i>M</i> -phosphate	4	45	4.6

Tissue metabolism

The methods described by McIlwain & Grinyer (1950) were followed for cutting and handling tissue slices, preparation of saline solutions, determination of phenosafranine, measurement of respiration and lactic acid formation, and manometric determination of the course of acid formation. When cozymase was determined in slices after incubation in fluids without added cozymase or dihydrocozymase, the slices (usually 0.35 mm. thick and of 50–60 mg. moist weight) were picked from the fluids with a mounted, bent platinum wire, drained against the side of the vessel and dropped into 1 ml. of the heated extracting solution. The cozymase content of the fluid used in incubation, under these conditions, has been found to be extremely small, although that of the tissue may be well maintained. For determining the cozymase of slices which had been incubated with cozymase and dihydrocozymase, the same procedure was followed if the initial cozymase level of the solution was not much greater than that expected in the slice. When the initial cozymase or dihydrocozymase concentrations were much higher than those in the slice, the slice was transferred to about 10 ml. of 0.9% NaCl for 1–2 sec. (ensuring that it opened in the saline) and was then drained and dropped into the extracting fluid. Homogenates were made in test-tube homogenizers from the whole cerebral hemispheres, with three times their volume of suspending fluid. Weights of tissue, when not otherwise specified, refer to fresh weights or those immediately after dissection. When slices of tissue were weighed after cutting or placing in saline, and then draining from the saline, the result is referred to as a moist or wet weight.

portion of cerebral hemisphere, approx. 0.1 g., was weighed as rapidly as possible on a torsion balance, dropped into phosphate buffer pH 6.2 containing nicotinamide at 100°, broken up, and the whole cooled after 2 min. The cozymase content of the guinea pig cerebral hemisphere averaged 159 μg./g. wet weight. Lower values for cozymase were obtained under other conditions. Thus, when the concentration of nicotinamide in the extracting fluid was 0.04*M*, the yield of cozymase was only 100 μg./g. Extraction in water for 2 min. at 100° yielded only 8 μg. cozymase/g. fresh weight.

Table 3. *Content of cozymase and of total nicotinic acid in brain*

Preparation	Nicotinic acid (μmol./g. fresh wt.)	Cozymase (μmol./g. fresh wt.)
Rabbit cerebral hemisphere	0.204	0.153
Guinea pig cerebral hemisphere	0.275	0.257
Rat whole brain	0.260	0.240
Mouse whole brain	0.590	0.505

It has been necessary to consider carefully the adequacy of this method of preparing the tissue for analysis, in view of the rapid degradation of cozymase in damaged tissue. For this purpose, the levels of cozymase have been compared with those of the total nicotinic acid of the same tissue preparations

(Table 3). The values showed that cozymase, as extracted by the present method, accounted for 93 % of the total nicotinic acid in guinea pig brain. Corresponding values were: rat 92, mouse 85, and rabbit 75 %. Breakdown of the nicotinamide nucleus during these experiments is considered unlikely, and at least in the case of guinea pig and rat, therefore, the method of preparation of the specimen does not lead to significant loss of cozymase.

Cozymase in different animals. Species differences in the cozymase content of brain tissue have been observed by Axelrod, Elvehjem & Spies (1941), and in the total nicotinic acid content of brain after enzymic hydrolysis by Taylor, Pollack & Williams (1942). The cozymase content of guinea pig cerebral hemispheres found by us (159 $\mu\text{g./g.}$) is greater than the value given by Axelrod *et al.* (1941) (107 $\mu\text{g./g.}$),

Homogenates prepared from guinea pig cerebral hemispheres in 0.1 M-nicotinamide gave cozymase contents corresponding to 127 $\mu\text{g./g.}$ fresh weight (Table 4). This level fell to 86 $\mu\text{g./g.}$ if the homogenate was kept at 20° for 15 min. In the absence of nicotinamide the cozymase level in a tissue homogenate dropped, by the time it could be pipetted into extracting fluid, to approximately 25 % of the cozymase present in fresh tissue. Following this, the cozymase content of the homogenate remained relatively constant for at least 30 min. The nature of the fluid in which the homogenate was prepared affected the level to which the cozymase concentration fell. It appeared that the residual cozymase might be bound in some subcellular structure, protected from attack by the cozymase-splitting enzyme of brain but liberated by the extraction.

Table 4. *Cozymase in brain homogenate*

(Guinea pig cerebral hemispheres were homogenized in 3 vol. liquid, and then extracted at the times stated in nicotinamide (0.1 M final conc.) in 0.1 M-phosphate buffer pH 6.2 at 100°.)

Homogenate kept at 20° prior to extraction for (min.)	Cozymase content ($\mu\text{g./g.}$ fresh wt.) after homogenizing in			
	0.1 M-Nicotinamide	Physiological saline (Krebs & Henseleit, 1932)	0.9 % NaCl	Water
0	127	—	36, 52	—
5	115	—	46, 52	—
10	—	—	46, 49	—
15	86	—	52	—
20	—	—	42	—
30	—	45, 70	30, 41	10, 30

while the value found by us for rat-brain cozymase content (156 $\mu\text{g./g.}$) was less than half the value given by Axelrod *et al.* (1941) or that corresponding to the total nicotinic acid level in rat brain given by Taylor *et al.* (1942). Taylor *et al.* used a Wistar strain of rats; those used by us were Norwegian 'hooded' rats. This difference in strain may account for the difference in cozymase level. The total nicotinic acid content of mouse brain recorded by Taylor *et al.* (1942) corresponds closely to our value for cozymase in mouse brain (333 $\mu\text{g./g.}$). It is observed that with the four species which we have examined the cozymase content of the brain tissue seems to decrease with increase in brain size.

Cozymase in slices and homogenates. Cozymase was found to be relatively stable in guinea pig cortex slices. When slices approximately 0.35 mm. thick and 50 mg. in weight were extracted immediately on section, their cozymase content ranged from 135 to 176 $\mu\text{g./g.}$ wet weight with an average of 152 $\mu\text{g./g.}$ wet weight. Tissue slices which had been placed, as they were cut, into an aerated bath of Krebs-Ringer phosphate-glucose medium for 5 min. prior to use in an experiment had cozymase contents of 82–132 $\mu\text{g./g.}$ wet weight (average 109 $\mu\text{g./g.}$).

Cozymase content of slices during in vitro metabolism

The preceding experiments suggested that slices could be cut from brain cortex with little loss of their cozymase. Subsequent to this, however, even leaving in saline for short periods caused some loss. To study the maintenance of cozymase during typical metabolic procedures, slices were placed, as cut, in aerated saline solutions containing various substrates. After cutting sufficient slices for an experiment, one after another of them was drained from the saline and weighed on a torsion balance. Some were transferred immediately to the nicotinamide-phosphate at 100°, and others put into saline solutions of different substrates, in the main compartments of Warburg vessels. Gas exchange was followed at 37°, and after different intervals slices were removed from the vessels with a mounted bent platinum wire and transferred to the extracting fluid at 100°.

Cozymase was found to be relatively stable during the first 70–90 min. under a variety of conditions (Table 5). Aerobically, with glucose in phosphate saline its level fell to about two-thirds of the initial

value in the first 80 min. The fall was not significantly greater if the substrate was omitted; the slice continued to respire, in the absence of substrate, at about half the rate of its respiration in the presence of glucose. Pyruvate and glutamate maintained similar levels of respiration and of cozymase. In several experiments the cozymase of slices was compared after incubation (a) anaerobically and without added glucose, and (b) aerobically with glucose. Cozymase levels were found to be lower in the absence of glucose and oxygen.

When slices, whose cozymase content had been lowered in this way, were further incubated aerobically and with glucose or glutamate, their cozymase continued to fall (Table 5, Exp. 4); their rate of respiration was also low. Slices pretreated in this way and then incubated anaerobically with glucose also continued to lose cozymase; their rate of glycolysis was less than 5% of that of fresh slices and their cozymase content about 40%.

decrease in the cozymase of the slices. Effects remained small in phosphate and bicarbonate buffers during aerobic reaction with glucose, glutamate, pyruvate, and a mixture of pyruvate and fumarate. Some anaerobic reactions were also studied (Table 5). The change produced by phenosafranine was, however, consistently a fall in cozymase level. During the present experiments, phenosafranine was absorbed by the slices as has been described by McIlwain & Grinyer (1950).

Addition of cozymase and dihydrocozymase to slices.

In considering how the normal cozymase content of brain tissues might be maintained, assimilation of cozymase was examined (Table 6). When slices as ordinarily prepared for metabolic studies were incubated under various conditions, the cozymase level of those incubated in the presence of added cozymase differed little if at all from those incubated in its absence. Moreover, if slices were first depleted in cozymase by anaerobic incubation, and cozymase

Table 5. *Cozymase of guinea pig brain cortex slices after metabolism in presence and absence of phenosafranine*

(Results similar to those of Exp. 2 were obtained in the absence of substrate and results similar to those of Exp. 3 were obtained with pyruvate (0.017M) and with pyruvate and fumarate as substrates. Respiration during the aerobic experiments was of the order of 70–95 $\mu\text{mol./g. moist wt./hr.}$ and was little (less than 10%) affected by phenosafranine. Phenosafranine, however, greatly increased aerobic acid formation from glucose.)

Exp. no.	Metabolic conditions (O ₂ , aerobic; N ₂ , anaerobic)	Period of metabolism (min.)	Cozymase content of slices ($\mu\text{g./g. moist wt.}$) after metabolism in	
			Medium alone	Medium with 10 ⁻⁵ M-phenosafranine
1	Phosphate; glucose; O ₂	0	171	—
		40	128	134
		80	114	87
2	Bicarbonate; glucose; O ₂	0	120	—
		90	79	57
	Bicarbonate; glucose; N ₂	90	61	60
3	Bicarbonate; glutamate; O ₂	0	120	—
		75	85	74
	Phosphate (no substrate); O ₂	0	120	—
		75	82	—
	Phosphate; glutamate; O ₂	0	120	—
		75	89	77
4	After initial anaerobic incubation in phosphate, no substrate, anaerobically	0	64	—
		60	26	—
	As above, with glutamate	30	30	21
		60	26	36

Phenosafranine. Although this substance (see below, p. 127) causes large changes in the aerobic carbohydrate metabolism of brain-cortex slices, it did not have a large effect on their cozymase levels. In Table 5, Exp. 2 illustrates this: a concentration of phenosafranine which increased aerobic lactic acid formation three- to six-fold, caused a small

added later, aerobically or anaerobically, little additional cozymase could be detected in the slice (Exp. 3, Table 6).

Assimilation of dihydrocozymase was also examined as it is stable in the presence of the degrading enzyme of brain and also it is less polar than cozymase. Results, however, were similar (Table 6). In

Table 6. *Cozymase content of slices after incubation in the presence of cozymase and dihydrocozymase*

(Slices (weighed) of 50–70 mg. were incubated at 37° in 3 ml. of saline containing the stated weights of coenzyme. When two periods of incubation were arranged, under different conditions, these followed within 1–5 min. of each other. Results similar to those of Exp. 1 were obtained after 180 min. incubation, and similar to those of Exp. 2 after preliminary incubation anaerobically in phosphate-glucose, and with the addition of either Co I or Co I H₂.)

Exp. no.	Conditions of incubation		Cozymase of slice (μg./g. moist wt.)		Duration of second period (min.)
	First period	Second period	At beginning of second period	At end of second period	
1	None	Phosphate-glucose; air	120	67	90
		Phosphate-glucose; Co I H ₂ , 670 μg.	120	82	90
2	Bicarbonate-glucose; N ₂ ; 140 min.	Bicarbonate-glucose; N ₂	48	37	60
		Co I H ₂ , 27 μg.	48	40	60
		Co I H ₂ , 134 μg.	48	48	60
3	Phosphate-glucose; N ₂ ; 120 min.	Phosphate-glucose; air	42	42	20
			42	35	60
		Phosphate-glucose; Co I H ₂ , 100 μg.	42	41	20
			42	34	60

experiments (2) and (3) of Table 6, most of the dihydrocozymase added was present in the fluid in which the slices had been incubated. It had, therefore, not been oxidized and degraded, or otherwise changed, but nevertheless it was not assimilated into the slices.

Attempts to increase cozymase content of slices by adding possible precursors. Attempts have been made to obtain a synthesis of cozymase in brain slices by incubating them in a medium containing mixtures of various constituents of the cozymase molecule. Kohn (1938) and Axelrod *et al.* (1941) had observed an increase in the cozymase content of red blood cells of pellagrins while they were being treated with nicotinic acid, and Kohn & Klein (1939, 1940) obtained a synthesis of cozymase in red blood cells *in vitro* by incubating them in a medium containing nicotinic acid. The acid, in this respect, was superior to the amide. For this reason nicotinic acid, as well as the amide, has been included in our reagents. Guinea pig brain slices were incubated aerobically at 37° for 30 or 120 min. in physiological salines buffered at pH 7.4 with 0.02M-phosphate, containing 0.01M-glucose, with and without the addition of 0.067M-nicotinamide, and 0.0033M-nicotinic acid, ribose and adenosinetriphosphate. No consistent evidence of synthesis was obtained when cozymase was determined by the apozymase system.

In other experiments, so that a small synthesis of cozymase might be more easily detected, the tissue slices were incubated for 2.5 hr. anaerobically in the saline containing 0.01M-glucose to reduce their original cozymase content. The average cozymase content of the tissue slices after this treatment was 45 μg./g. wet weight. After this anaerobic incubation of guinea pig slices, nicotinamide, nicotinic acid, ribose and adenosinetriphosphate or

adenylic acid were added to the medium in final concentrations of 0.0033M and aerobic incubation was allowed to proceed for 40 min. There was no detectable synthesis of cozymase in the tissue slices under these conditions nor when the experiment was repeated with 0.1M-nicotinamide. This experiment was also carried out anaerobically in the presence of 0.01M-glutathione with negative results.

Sodium glutamate (0.01M) was used in place of glucose in two experiments. In addition to glutamate, the medium contained 0.1M-nicotinamide and 0.0033M-adenylic acid. The experiment was made both with fresh tissue slices and those in which the cozymase had been allowed to fall anaerobically, but no synthesis of cozymase was detected.

DISCUSSION

The present observations have partly answered the questions posed by the coexistence in brain of cozymase and a system which rapidly degrades it. The brain of guinea pigs or rats could be removed from the animal at room temperature and sampled (operations lasting 2–5 min.) without loss of more than 10% of its cozymase. This was judged from the finding that the content of cozymase was about 92% of the content of nicotinic acid. The maximal loss is thus some 10%; some nicotinamide may normally exist in the tissue as such and some certainly exists as coenzyme II. On the other hand, in homogenates made at 0° the cozymase content fell to 20% of the initial content of the tissue, within 2 min. The stability of cozymase in slices of the sort employed in studies of tissue metabolism was very much greater: some 60–70% remained after slicing and floating in physiological salines, operations which occupied 5–10 min. On incubating aerobically under normal

manometric conditions an hour or so was needed for the level of cozymase in the slices to fall to 50 % of that in the fresh whole brain.

Anaerobically, the cozymase of slices was depleted more rapidly, and conditions have not yet been found for its restoration. No evidence was found that cozymase was assimilated into either normal or depleted slices, or that dihydrocozymase, which was stable when added to slices and which might be considered more likely to penetrate them, was assimilated. Various mixtures containing nicotinamide, nicotinic acid, and adenine derivatives did not lead to increased cozymase. Examination of the possible route of synthesis is being continued. Differential determination of the oxidized and reduced forms of cozymase has not been attempted in the present study; the two were determined together by the apozymase system.

It was interesting to determine whether phenosafranine, which both increased the aerobic formation of lactic acid by brain-cortex slices (Dickens, 1936) and inhibited the degradation of cozymase in brain homogenates (McIlwain, 1949), affected the cozymase level of brain slices. The results showed that phenosafranine induced a slightly greater fall in the cozymase content of slices than that occurring in its absence. If the correlation suggested by McIlwain & Grinyer (1950) between the action of agents on cozymase degradation and on aerobic glycolysis reflects a real linkage between the two processes, then it is necessary to consider that the system, which in homogenates degrades cozymase, is functioning in slices without leading to loss of cozymase. This, and other observations of this and the preceding paragraph which are not yet understood, may be explained in terms of the complexity

of the degradation and of other reactions undergone by cozymase.

SUMMARY

1. A method has been developed for determining the cozymase content of brain tissue.

2. The cozymase content of the cerebral hemispheres of mouse, rat, guinea pig and rabbit brain were determined. The values obtained were: mouse, 333, rat 158, guinea pig 159, rabbit 103 $\mu\text{g./g.}$ fresh tissue respectively. The cozymase contained in guinea pig brain represents at least 92 % of the total nicotinic acid in the tissue.

3. Cozymase was found to be relatively stable in brain slices. There was an average fall of 28 % of the initial cozymase content of the tissue 10 min. after section and a much slower fall during active metabolism. The destruction of cozymase in homogenized tissue was very rapid. Only 25 % of the initial cozymase remained 2 min. after homogenizing brain tissue in saline. The residual 25 % cozymase seemed to be protected from the action of the enzyme degrading cozymase.

4. The increased glycolysis in brain-tissue slices produced by phenosafranine was not due to the maintenance of a high level of cozymase in the slices. In the presence of phenosafranine the fall in cozymase was rather greater than in its absence.

5. Attempts to synthesize cozymase in brain tissue slices by incubation with various components of the cozymase molecule did not lead to an increase in their coenzyme content, nor did incubation with cozymase and dihydrocozymase.

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