in diffusion currents corresponding to the lead in 20 ml. urine. Concentrations of lead smaller than 50 $\mu\text{g./l.}$ can, however, be determined by the modified procedure reported at the end of the experimental section.

No attempt has been made to use the present methods for material other than urine.

SUMMARY

1. Two relatively rapid methods for the polarographic determination of pathologically increased lead in urine are described, together with a modification for the estimation of lower concentrations.

2. The quantitative recovery obtained in the

entrainment procedures of Ross & Lucas (1935) is confirmed.

3. Applications to cases of suspected lead intoxication are described; there is evidence that 2:3-dimercaptopropanol (BAL) increases the excretion of lead in the urine.

4. Estimates have been obtained for the diffusion coefficients of the complex lead ion in citrate buffer in presence and absence of calcium.

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Properties of Preparations from the Central Nervous System which Degrade Coenzymes I and II; their Connexion with Carbohydrate Metabolism

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Extraction of the system which degrades cozymase in nervous tissues, and examination of the action of various agents upon it, were considered to be of value as the system is a very potent one which acts also on coenzyme II though not on the dihydrocoenzymes (McIlwain, 1949*a, b*). It could thus affect the main energy-yielding reactions of the brain at several points.

The system has previously been examined in brain slices and in ground brain (for references see McIlwain & Rodnight, 1949*a, b*). In such preparations a large part of the activity remains firmly

attached to the solid particles. There is also evidence that material with cozymase-degrading activity passes into solution in homogenates of brain in water (Utter, Wood & Reiner, 1945; Meyerhof & Wilson, 1947; McIlwain & Rodnight, 1949*a*). We have found that the active material is also soluble in strong salt solutions, and that this property, combined with its solubility in water and relative insolubility in isotonic saline, can be used to separate it from other materials.

Following the separation of the cozymase-degrading system, an examination has been made

of the action on it of various substances which affect either carbohydrate metabolism or the functional activity of nervous tissues. These studies have suggested that the system degrading cozymase is involved in the control of aerobic glycolysis.

EXPERIMENTAL

Cozymase specimens, salines, tissue preparations, and methods of determining nicotinamide and acid formation during cozymase breakdown were those previously described (McIlwain & Rodnight, 1949*a, b*).

Examination of cozymase-degrading activity during fractionation of the enzyme was made anaerobically at pH 7 in bicarbonate-NaCl as described by McIlwain & Rodnight (1949*a*), CO₂ evolution and nicotinamide formation being measured. From the manometric readings, the initial rate of cozymase degradation was determined graphically. The amount of material taken for estimation was so arranged that the rates measured were between 0.8 and 3 $\mu\text{mol./vessel/hr.}$ Total N was determined by a micro-Kjeldahl method.

The action of various substances on cozymase degradation was assessed by adding them, together with the degrading enzyme in bicarbonate saline, to the main compartments of Warburg vessels. The side arms contained cozymase, the centre well yellow phosphorus, and the gas space 5 % (v/v) CO₂-N₂ except when otherwise described. The initial concentration of cozymase in the reaction mixture was between 4 and 6 $\times 10^{-4}\text{M}$. The rate of reaction in most cases was followed by evolution of CO₂ and checked by determination of nicotinamide at the end of reaction. When the course of the reaction could not be followed by CO₂ evolution (some reagents retained CO₂, and when CO was studied, the reaction was carried out in phosphate-saline), more than one determination of nicotinamide was made in order that the rate of breakdown could be obtained.

Of the heterocyclic compounds whose actions were studied, pyridine-3-sulphonamide was prepared according to Machek (1938); dihydrocozymase (McIlwain & Rodnight, 1949*b*) according to Schlenk (1941); phenazine methosulphate, as described by Dickens & McIlwain (1938); 2-aminophenazine methosulphate, according to Kehrmann (1914); pyocyanine, according to McIlwain (1937). Nicotinic acid hydrazide was given by Dr F. L. Rose, Imperial Chemical (Pharmaceuticals) Ltd.; 3-acetylpyridine by Dr M. Gore (prepared according to Strong & McElvain, 1933); 4(5)-3'-pyridylglyoxaline by Dr G. C. Leitch (Clemo, Holmes & Leitch, 1938); the nicotinamide glycosides by Prof. A. R. Todd and Dr J. L. Haynes; ethylred by Dr F. M. Hamer, Kodak, Ltd.; and lysergic acid diethylamide by Sandoz Products, Ltd.

RESULTS

Extraction and purification

Extraction with water. This is shown for sheep brain and ox spinal cord in Table 1.

In preparation A, sheep brain (80 g.) was rubbed for 5 min. in a mortar with an equal weight of sand, and suspended in 400 ml. 0.9 % NaCl. This was centrifuged and the residue resuspended in saline by mechanically stirring for 3 min., and again centrifuged. Three such washings were given in all, and the residue made up to 760 ml. with the saline.

Table 1. *Extraction of cozymase-degrading enzyme with water*

Starting material	Preparation examined	Activity in degrading cozymase	
		$\mu\text{mol./hr./specimen derived from 1 g. fresh weight}$	$\mu\text{mol./hr. mg. N}$
A. Sheep brain	Ground	145	8.5
	1st water extract	21	14.5
	2nd water extract	60	17.0
	3rd water extract	12	14.9
	Residue from extraction	3	7.6
B. Ox spinal cord	Ground and washed with 0.9 % NaCl	57	11.6
	1st water extract	1.6	7.4
	2nd water extract	4.9	4.5
	3rd water extract	21	24.4
	Residue	19.2	8.3

For extraction, 150 ml. of this suspension were centrifuged, the solid stirred with 150 ml. of water, and again collected by centrifuging. This solution, still containing saline from the original preparation, was relatively clear and had little activity. Continued washing now yielded more active, opalescent extracts, and in the preparation A of Table 1, about half the activity of the original preparation is seen to be removed by two washings. Most of the activity not accounted for (about one-third of the whole) was in the first saline washings which were discarded, and of which the activity/unit volume was small. Other preparations of brain (sheep and guinea pig), in which the volumes of washing and extracting fluids and the times of extraction were varied, gave results which differed in detail from those of Table 1, but in which the proportion and activity of the extracted material were comparable. Active material was extracted also from ox cord by this method, but somewhat less readily than from brain (Table 1).

Properties of the aqueous extracts. These were opalescent solutions containing about one-third of the nitrogen of the original preparation. Their preparation was therefore not an important step in purification. They contained no structures which could be recognized microscopically at a magnification of 1000, in distinction to the brain ground with sand in which much tissue debris, nuclei and granules were visible. The extracts retained activity with little loss during 1 month in a refrigerator. During this time, material containing about one-third of both the nitrogen and the activity precipitated spontaneously. The bulk of the remaining nitrogen and activity could be precipitated by mild acidification. Attempts to base separations on this property were not successful; acetate buffers of pH between 4 and 6 precipitated varying amounts of the solid and activity without significant enrichment.

Precipitation from aqueous solution by ethanol resulted in partial loss of activity; with acetone

neither loss nor enrichment was found. After keeping, some precipitation occurred with phosphate (0.02M, pH 7), with a little enrichment (29 $\mu\text{mol./hr./mg. N}$; starting material, 21 $\mu\text{mol./hr./mg. N}$), but including a variable part of the initial activity. Active material was easily precipitated by sodium sulphate (80 % precipitated at 0.75M, pH 7.5; 73 % precipitated at 0.27M with 0.02M phosphate, pH 7; activity, 25 $\mu\text{mol./hr./mg. N}$), but again a useful separation was not obtained. Preparation *WS* of McIlwain & Rodnight (1949b) was obtained in this method.

Extraction with 1-1.5M-sodium chloride. In contrast to the extraction with water, this process yielded fractions of markedly increased activity. It was carried out because the extraction with water, and the properties of the water extract, suggested that the activity of the preparation was associated with nucleoprotein-like materials. Whether this is correct or not, it was found that after nervous tissue had been washed with isotonic saline, with the removal of some cozymase-splitting activity, extracts containing more of such activity could be obtained by washing with 1.2M-NaCl (Table 2). A proportionately smaller quantity of nitrogenous

extracted by stirring with 1.2M-NaCl (1 l.) for 30 min. at 10°, centrifuged, and the residue treated again in the same way. For preparation *C*, 200 g. cord were blended (Waring) for 12 sec. with 200 ml. 0.9 % NaCl, a further 800 ml. added, and the mixture stirred for 15 min. After centrifuging, the washing was twice repeated; extraction with 1.2M-NaCl was done as above.

Properties of sodium chloride extracts. In order to test for cozymase degradation, most of the sodium chloride was removed by dialysis in cellophan bags against rapidly running tap water, at 10° for 2 hr. During this time, inactive material precipitated. Relatively little nitrogenous material passed out of the bags; this is understandable, as prior to extraction the tissue had been well washed with 0.9 % sodium chloride. No morphological structure was visible in the final preparations. These constituted the preparations *SaDC* referred to by McIlwain & Rodnight (1949b).

Some enzymic properties of preparations degrading cozymase

Many enzymes of brain tissue are lacking in the washed ground brain or cord used as the source of the degrading enzyme, for the reaction with cozymase could be followed satisfactorily by manometric methods in the presence of lactate, pyruvate, glucose, glutamate, malate, or ethanol. These compounds therefore underwent no changes leading to changes in acid or gas under the conditions of determining cozymase breakdown.

Enzymes hydrolysing adenosine triphosphate are, however, known to be associated with particulate matter in brain homogenates (Meyerhof & Wilson, 1947). Because of their relevance to carbohydrate metabolism, we have determined the rate of breakdown of adenosine triphosphate by preparations degrading cozymase (Table 7). Washed brain preparations catalysed a rapid breakdown of adenosine triphosphate with formation of inorganic phosphate, loss of acid-labile phosphate, and formation of acidic groupings which liberated some carbon dioxide from bicarbonate. Their ability to do this was, however, almost entirely lost during the extraction with strong salt and the dialysis, by which the cozymase degrading system was concentrated (Table 7).

Effects of substances on cozymase degradation

General. The actions of several substances have been assessed quantitatively with different preparations of the degrading enzyme. The action of nicotinamide on cord preparations which were simply washed with 0.9 % sodium chloride was compared with its action on an extracted and dialysed enzyme from the same source. No marked difference was found (Table 3). The action of phenosafranine on the activity of a cord preparation was similar to that of its action on the activity of an extract from

Table 2. *Extraction of cozymase-degrading system with 1.2M-sodium chloride*

Starting material	Preparation examined	Activity in degrading cozymase	
		$\mu\text{mol./hr./specimen derived from 1 g. fresh weight}$	$\mu\text{mol./hr./mg. N}$
A. Guinea pig brain	Ground	210	13.0
	3 washings with 0.9 % NaCl	25	3.3
	1st 1.2M-NaCl extract	44	25
	2nd 1.2M-NaCl extract	29	30
	Residue	72	16.5
B. Ox spinal cord	Ground and washed with 0.9 % NaCl	102	12.0
	0.9 % NaCl washings	47	21.3
	1st 1.2M-NaCl extract	14	100
	2nd 1.2M-NaCl extract	8	106
	Residue	22	4.3
C. Ox spinal cord	Blended	120	10.4
	1st 1.2M-NaCl extract	22	72
	2nd 1.2M-NaCl extract	20	129
	3rd 1.2M-NaCl extract	6	32
	Residue	20	5.4

material was extracted, and the activity of the preparations in $\mu\text{mol. cozymase split/hr./mg. N}$ therefore rose. In this respect, spinal cord was a more satisfactory starting material than brain; from cord, preparations with activities of 100-150 $\mu\text{mol. cozymase split/hr./mg. N}$ were obtained.

For preparation B, Table 2, ox cord, brought from a slaughter-house in ice, was passed through a cold meat mincer, rubbed with sand as above, and passed through muslin to remove membranes. It was suspended in 1 l. 0.9 % NaCl, stirred for 5 min., centrifuged, and the residue resuspended and washed twice as before. The residue was then

brain (Table 5). The actions of potassium and calcium chlorides, the pyridylglyoxaline, neutral red, and phenosafranine were also examined on a variety of preparations which included specimens from guinea pig, rabbit, and sheep brains, and no major differences were found which were specific to the preparations.

Table 3. *Inorganic compounds and pyridine derivatives on cozymase degradation*

Substance	Concentration in reaction mixture (M)	Enzyme preparation*	Rate of reaction (% of rate without added substance)
KCl	10^{-1}	C, SsDC	96
CaCl ₂	2×10^{-2}	C, SsDC	100
NaF	33×10^{-3}	B, W	105
KCN	33×10^{-3}	B, W	100
K ₃ Fe(CN) ₆	1.67×10^{-3}	B, W	106
K ₄ Fe(CN) ₆	1.67×10^{-3}	B, W	96
KI	$3.3, 0.67 \times 10^{-3}$	B, W	107, 102
Nicotinamide	2.67×10^{-3}	B, W	27
Nicotinamide	1.33×10^{-3}	B, W	44
Nicotinamide	6.67×10^{-4}	B, W	60
Nicotinamide	3.33×10^{-4}	B, W	90
Nicotinamide	1.33×10^{-3}	C, E	42
Nicotinamide	6.67×10^{-4}	C, E	54
Nicotinamide	3.33×10^{-4}	C, E	78
Nicotinic acid	3.3×10^{-3}	C, SsDC	108
hydrazide			
Coramine	3.3×10^{-3}	B, W	95
3-Acetylpyridine	1.1×10^{-2}	C, SsDC	35
3-Acetylpyridine	1.7×10^{-3}	C, SsDC	100
Pyridine-3-sulphonamide	8.3×10^{-3}	B, W	104
Nicotine	1.67×10^{-2}	B, W	24
Nicotine	1.67×10^{-3}	B, W	47
Nicotine	1.67×10^{-4}	B, W	65
4(5)-3' Pyridylglyoxaline	8.3×10^{-3}	B, W	<5
4(5)-3' Pyridylglyoxaline	1.67×10^{-3}	B, W	5
4(5)-3' Pyridylglyoxaline	3.3×10^{-4}	B, W	14
4(5)-3' Pyridylglyoxaline	10^{-4}	B, W	53
4(5)-3' Pyridylglyoxaline	3×10^{-5}	B, W	79
4(5)-3' Pyridylglyoxaline	10^{-5}	B, W	95
Trigonelline chloride	8.3×10^{-3}	B, W	105
Nicotinamide methiodide	3.3×10^{-3}	C, W	97
Nicotinamide riboside	3.3×10^{-3}	C, W	85
Nicotinamide glucoside	3.3×10^{-3}	C, W	78
Dihydrocozymase	4×10^{-4}	B, W	98

* B, brain from guinea pig or sheep; C, ox spinal cord; W, washed with 0.9 % NaCl; E, extracted with water; SsDC prepared by extraction with strong salt, dialysed and centrifuged. Details of preparation are given in the previous part of this paper. Differences of less than 6 % in the rates are not considered significant.

Inorganic salts. The effects of Ca⁺⁺ and K⁺ were examined because these ions affect nervous activity, and Ca⁺⁺ had been reported by Swingle, Axelrod & Elvehjem (1942) to accelerate cozymase breakdown in homogenized rat liver, though Spaulding & Graham (1947) did not find an action in ground brain. No effect of Ca⁺⁺ and K⁺ had previously been found in this laboratory on cozymase degradation in sodium chloride washed ground sheep brain, and no action has now been found (Table 3) on the breakdown by extracted enzyme. In addition to the experiment of Table 3, the effect of adding potassium chloride (0.1 M) to a reaction mixture in a balanced physiological saline was examined, but again no change was caused. This concentration of potassium chloride causes full release of aerobic glycolysis in brain (Ashford & Dixon, 1935; Dickens & Greville, 1935).

Fluoride and cyanide ions, also found to be without action on cozymase degradation, were examined since they are frequently used as reagents. It was necessary to know the effects of iodides and of ferri- and ferro-cyanides (all found inactive) in order to control experiments in which these reagents were employed.

3-Substituted pyridine derivatives. Inhibition by nicotinamide is already well known (Mann & Quastel, 1941). From Table 3 it is seen to be representative of the inhibitory powers of certain 3-substituted pyridine derivatives which include nicotine, 4(5)-3'-pyridylglyoxaline (for structures, see Table 4) and 3-acetylpyridine. Of these compounds, 3-acetylpyridine was not a potent inhibitor; it would be necessary for the compound to be greatly concentrated in tissues if the present inhibition is the basis of the growth effects observed by Woolley (1945). Both nicotine and nicotinamide were more active, inhibiting the breakdown of 6×10^{-4} M-cozymase by 50 % at about 10^{-3} M. The pyridylglyoxaline was much more potent as an inhibitor, producing comparable effect to nicotinamide at about 0.1 of the concentration.

Relatively small changes in the structure of nicotinamide produced substances of greatly reduced potency. Thus, nicotinic acid, pyridine-3-sulphonamide, and nicotinic acid hydrazide and its diethylamide (coramine) showed no activity at concentrations equal to or greater than that at which nicotinamide reduced the reaction to 20 % of its uninhabited velocity. Quaternary salts of nicotinamide were also less active: nicotinamide methiodide very much so, while the riboside and glucoside had relatively small activities which were not clearly differentiated from that due to nicotinamide in the preparations. These relationships between structure and activity are to be contrasted with those of phenazine derivatives, among which quaternary salts were the more potent inhibitors. It was noted

Table 4. *Some compounds whose action on cozymase degradation has been examined*

Substance	Basic Formula	Substituents in position				
		2	3	6	7, 8	10
Neutral red	(IV)	Me	NH ₂	NMe ₂	—	—
Neutral violet	(IV)	NH.C ₆ H ₄ NMe ₂	NH ₂	NMe ₂	—	—
Phenosafranine	(IV)	—	NH ₂	NH ₂	—	C ₆ H ₅ .Cl
Janus green	(IV)	—	N ₂ C ₆ H ₄ NMe ₂	NH ₂	—	C ₆ H ₅ .Cl
Rosinduline	(IV)	—	—	NH ₂	C ₆ H ₄	C ₆ H ₅ .Cl
Methylene blue	(V), X=S	—	NMe ₂ .Cl	NMe ₂	—	—
Thionine blue	(V), X=S	—	NMeEt.Cl	NMe ₂	—	—
Brilliant cresyl blue	(V), X=O	—	NH ₂ .Cl	NEt ₂	—	—

previously (McIlwain & Rodnight, 1949b) that dihydrocoenzymes I and II had little effect on the breakdown of cozymase, while coenzyme II competed with coenzyme I.

Alkaloids with fused pyridine or piperidine rings. Many such substances were examined without finding a potent inhibitor. The relatively small activities of quinine and strychnine are recorded in Table 6. Cinchonidine, atropine, emetine, and bulbocapnine at 3×10^{-3} M were without action on the breakdown catalysed by washed brain preparations. Lysergic acid diethylamide was similarly inactive at 3×10^{-4} M, and narcotine in a saturated solution in 0.9 % sodium chloride was also without action.

Phenazine derivatives and related compounds. Investigation of these compounds was centred round the observation (McIlwain, 1949a, b) that phenosafranine, a diamino-*N*-phenyl phenazonium salt, was a potent inhibitor of cozymase degradation. Phenosafranine had been chosen for examination on account of Dickens's (1936) observation of its release of aerobic glycolysis in brain-cortex slices and in other preparations. Related compounds were accordingly examined (Table 5; for structures see Table 4).

A simple phenazonium salt, phenazine methosulphate, was much less active. Introduction of an amino group, in α -aminophenazine methosulphate, increased the activity. The keto group of pyocyanine had little effect. The two amino groups of neutral red are in the same positions as those of phenosafranine, although neutral red lacks the quaternary phenyl group (it is also methylated); neutral red is more active than the preceding compounds, but less so than phenosafranine. Neutral violet, with an additional nuclear amino group, but still lacking the quaternary phenyl group, possessed activity of the same order as that of neutral red.

Rosinduline and Janus green are both, like phenosafranine, aminophenazonium salts. Rosinduline was less active than neutral red; it however contains only one amino group, and also a fused benzene ring which does not appear in phenosafranine. Janus green is produced from phenosafranine by diazotizing and coupling it with dimethylaniline; its activity was found to be only slightly less than that of phenosafranine.

Methylene blue, thionine blue, and brilliant cresyl blue possess, instead of the phenazine, oxazine or thiazine nuclei, substituted similarly to the phenaza-

zines studied but lacking a quaternary phenyl group. Their activities were not large, all being less potent than the aminophenazine methosulphate.

Table 5. *Action of some phenazine, thiazine and oxazine derivatives on cozymase degradation*

Substance	Concentration in reaction mixture (M)	Enzyme preparation (see Table 3)	Rate of reaction (% of rate without added substance)
Phenazine methosulphate	3.3×10^{-4}	C, W	88
Aminophenazine methosulphate	3.3×10^{-4}	C, W	56
Pyocyanine	10^{-3}	C, SsDC	80
Pyocyanine	10^{-4}	C, SsDC	88
Neutral red	1.67×10^{-3}	G, B, W	44
Neutral red	1.67×10^{-4}	G, B, W	58
Neutral red	3.3×10^{-5}	G, B, W	78
Neutral red	10^{-4}	C, SsDC	65
Neutral red	10^{-5}	C, SsDC	81
Neutral violet	3.3×10^{-4}	C, W	61
Phenosafranine	1.33×10^{-3}	B, W	16
Phenosafranine	3.3×10^{-4}	B, W	13
Phenosafranine	6.7×10^{-5}	B, W	38
Phenosafranine	1.3×10^{-5}	B, W	88
Phenosafranine	10^{-4}	C, SsDC	28
Phenosafranine	10^{-5}	C, SsDC	85
Janus green	10^{-4}	C, SsDC (a)	31
Janus green	10^{-5}	C, SsDC (a)	78
Janus green	3×10^{-4}	C, SsDC (b)	28
Janus green	10^{-4}	C, SsDC (b)	38
Janus green	3×10^{-5}	C, SsDC (b)	75
Janus green	10^{-5}	C, SsDC (b)	95
Rosinduline	3.3×10^{-4}	C, W	78
Methylene blue	3.3×10^{-4}	C, W	98
Thionine blue	5×10^{-4}	C, SsDC	70
Thionine blue	1.67×10^{-4}	C, SsDC	95
Thionine blue	5×10^{-5}	C, SsDC	103
Brilliant cresyl blue	10^{-3}	C, SsDC	55
Brilliant cresyl blue	10^{-4}	C, SsDC	100

inactive at concentrations much higher than those which affect glycolysis (Dickens, 1939). Ethyl red was inhibitory only in relatively high concentrations, and styryl 430 and benzylviologen little if at all in the range examined (cf. Dickens, 1936).

Table 6. *Action on cozymase degradation of some substances affecting aerobic glycolysis; miscellaneous compounds*

Substance	Concentration in reaction mixture (M)	Enzyme preparation (see Table 3)	Rate of reaction (% of rate without added substance)
Carbon monoxide	(in equilibrium with 1 atm. in gas phase)	C, SsDC	93
Guanidine	10^{-2} , 10^{-3}	C, SsDC	100, 104
Methylguanidine	10^{-2}	C, SsDC	70
Methylguanidine	10^{-3}	C, SsDC	100
Synthalin B	10^{-3}	C, SsDC	97
Synthalin B	10^{-4}	C, SsDC	102
Undecane diamidine	10^{-3}	C, SsDC	102
Undecane diamidine	10^{-4}	C, SsDC	95
Ethyl red	3.3×10^{-4}	C, W	38
Ethyl red	6.7×10^{-5}	C, W	104
Ethyl red	5×10^{-6}	C, SsDC	90
Ethyl red	10^{-5} , 5×10^{-6}	C, SsDC	96
Styryl 430	10^{-4} , 10^{-5}	C, SsDC	94, 99
Benzylviologen	10^{-4} , 10^{-5}	C, SsDC	97
2,4-Dinitrophenol	1.66×10^{-3}	C, SsDC	55
2,4-Dinitrophenol	1.66×10^{-4}	C, SsDC	65
Quinine	3.3×10^{-3}	B, W	89, 95
Strychnine	3.3×10^{-3}	B, W	75, 90
Benzimidazole methiodide	3.3×10^{-3}	B, W	99

Miscellaneous materials. Many naturally occurring materials were without action on the degradation catalysed by washed brain or cord: ethanol (3×10^{-3} M), lactate (3×10^{-3} M), pyruvate (5 and 20×10^{-3} M), malate (3×10^{-3} M), histamine (2×10^{-2} M), mescaline (8×10^{-3} M), eserine (3.3×10^{-3}), adenosinetriphosphate (5×10^{-3} M), insulin (13 units/ml.) and protamine (0.3 and 1 mg./ml.). Three specimens of normal human serum (1 ml./3 ml. reaction mixture) were without action on the breakdown by a washed-cord preparation, as also were two extracts from sheep brain (1 ml./3 ml.). These were prepared (a) by blending the brain with an equal volume of water, heating to 100° for 15 min., and centrifuging; (b) with sodium sulphate according to the method of Deutsch, Eggleton & Eggleton (1938). Metrazol (3.3×10^{-3} M) was also inactive.

Lack of action of phenosafranine on breakdown of adenosinetriphosphate by brain homogenates

Washed particles from brain homogenates react also with adenosinetriphosphate (p. 614). Phenosafranine has been found, however, to have no action on this process. Table 7 shows that any effect of 3×10^{-5} M-phenosafranine is within the limits of

It thus appeared that while some activity in inhibiting cozymase degradation was possessed by a variety of phenazine and structurally similar substances, activity was greatest in the phenazines. This activity was increased by basic substituents, and by forming quaternary salts. Sufficient analogues were not examined for the significance of the individual groups in phenosafranine to be assessed.

Other substances affecting aerobic phosphorylation or aerobic glycolysis in brain-cortex slices. Phenosafranine and nicotine are especially interesting in affecting both phosphorylation and glycolysis, and accordingly the behaviour towards cozymase degradation of other substances which affect these processes has also been examined (Table 6). Carbon monoxide, like cyanide, was without effect on the breakdown. The experiment with carbon monoxide used an atmosphere of 100 % carbon monoxide, and light was excluded from the experimental vessel. The guanidines and diamidines tested were also

Table 7. *Comparison of breakdown of adenosinetriphosphate and cozymase by preparations from nervous tissue; lack of action of phenosafranine on the reaction with adenosinetriphosphate*

(Reactions were carried out anaerobically as described for cozymase degradation in the experimental section, except that the reaction mixture was a balanced bicarbonate saline (Krebs & Henseleit, 1932). Labile phosphate was determined as the difference in inorganic phosphate (Lowry & Lopez, 1946), before and after heating for 7 min. in $N-HCl$.)

Preparation (see Table 3)	Additions ($\mu\text{mol./3 ml.}$)	Change in labile phosphate ($\mu\text{mol./ml.}$)	Period (min.)	CO_2 evolved from bicarbonate		
				($\mu\text{mol./vessel}$)	Period (min.)	Initial rate ($\mu\text{mol./vessel/hr.}$)
B, W	Adenosinetriphosphate, 18	1.39	60	1.34	60	3.76
B, W	Adenosinetriphosphate, 18 phenosafranine, 0.1	1.35	60	1.26	60	3.70
B, W	Cozymase, 1.5	—	—	0.65	80	0.8
C, SsDC	Adenosinetriphosphate, 18	0.2	120	0.08	120	0.05
C, SsDC	Cozymase, 1.5	—	—	0.5	120	1.0

measurement, whereas this concentration inhibits cozymase degradation in the same preparations by about 50 %. Phenosafranine at an initial concentration of $3 \times 10^{-4} M$ was also without effect on the breakdown of adenosinetriphosphate.

DISCUSSION

The outstanding finding of the present survey is of the potent action of phenosafranine on cozymase degradation. The effect of this substance was examined because its action in disturbing the normal course of carbohydrate breakdown in brain cortex had remained without explanation since being discovered by Dickens (1936). The disturbance caused by phenosafranine consists of an increase in aerobic glycolysis to values approaching anaerobic levels, without much effect on respiration. It thus constitutes a disturbance of the Pasteur effect. It is remarkable that Dickens suggested in 1936 that the action of compounds like phenosafranine might be through processes in which coenzymes, including cozymase, took part. Phenosafranine has now been found to be the most potent of a group of phenazine compounds in its action on the enzyme system degrading cozymase, but this property is shared by other compounds. When comparison was made (McIlwain, 1949*a*) between the actions of these compounds on the degradation of cozymase, and on release of aerobic glycolysis, some correlation appeared to exist. The correlation was, however, complicated by concentration gradients of the substances between solution and tissue and is being made the subject of separate studies.

At this point it was clear that valuable evidence on the possible connexion between aerobic glycolysis and cozymase degradation would be given, if entirely different types of substances affected both processes. It has now been found that such evidence already exists in observations of previous workers, though these have not before been interpreted in this

fashion; and the evidence has also been extended by the observations of the present paper.

Baker, Fazekas & Himwich (1938), in determining whether glucose oxidation in brain passed through lactic acid, searched for differential inhibitors of metabolism of the two substrates and found nicotine, nicotinic acid and nicotinamide to inhibit differentially. In addition, however, nicotinamide at $0.03 M$ is shown from Table 3 of that paper to have only a small action on the respiratory rate of slices of rat cerebral cortex, but consistently to increase their aerobic lactic acid formation. The authors, in the text, minimized this observation as other compounds were more potent in showing both this effect and the differential action in which they were interested. However, as stated previously, nicotinamide was later shown to inhibit cozymase degradation and the compound thus afforded a connexion between cozymase metabolism and aerobic glycolysis of the same type as that afforded by phenosafranine.

Moreover, it has been observed that the same correlation extended to nicotine. Baker *et al.* (1938) found considerable increase in aerobic glycolysis of cat cerebral cortex by $0.03 M$ -nicotine, and an inhibition of respiration of 28 %. With guinea pig-brain cortex, we find an increase in glycolysis with lesser inhibition of respiration. As shown above, nicotine inhibits cozymase degradation. The correlation also extended to 4(5)-3'-pyridylglyoxaline (McIlwain, 1949*b*) a substance which is structurally similar to nicotine, but whose action on either cozymase degradation or aerobic glycolysis had not previously been studied. The pyridylglyoxaline was found to be more potent than nicotine in its action on both these processes.

Reviewing the results on inhibition of cozymase degradation, it is seen that all effective inhibitors of the breakdown are reported to increase aerobic glycolysis. Many compounds affect neither process. The structural criteria for effective inhibition of

cozymase degradation were fairly exacting, as all the potent inhibitors of the reaction belonged to the groups of phenazine and pyridine derivatives, though many others were examined.

Although all substances inhibiting cozymase degradation released aerobic glycolysis, many substances reported to release aerobic glycolysis were without effect on cozymase degradation: potassium chloride, carbon monoxide and the guanidines and amidines were outstanding in this respect. This is a finding to be expected when the action of substances on an organized series of reactions involving a dozen or more enzymes is compared with their actions on a single process of the series. Whether the reactions of carbohydrate degradation and utilization, as they operate in tissues, involve the continual breakdown and resynthesis of cozymase is unknown; but the above evidence strongly indicates that the enzyme isolated from the tissue which degrades cozymase does play a specific role in carbohydrate metabolism in brain. The mechanism of this participation appears likely to be through an effect on the phosphorylation normally accompanying oxidation, for Judah & Williams-Ashman (1949) found that phenosafranine inhibits the phosphorylation accompanying pyruvate oxidation in rat-liver homogenates, and phenosafranine has now been found in these laboratories (Case & McIlwain, unpublished) to have a similar effect with brain.

SUMMARY

1. The system degrading cozymase in preparations of mammalian brain and spinal cord has been extracted by water or by strong salt solutions, and separated from some 90 % of the nitrogenous constituents of the tissues.

2. Of about sixty-five materials which were examined for their actions on the breakdown, none caused significant stimulation even with a washed and dialysed enzyme preparation.

3. Two main groups of compounds inhibited the breakdown when present in concentrations comparable to, or less than, those of the cozymase undergoing reaction. These were pyridine and phenazine derivatives.

4. The active pyridine compounds included nicotine and 4(5)-3'-pyridylglyoxaline as well as nicotinamide. Of the phenazine derivatives, phenosafranine and Janus green were most potent.

5. Correlations existed between the actions of these substances on cozymase degradation and their reported actions on the release of aerobic glycolysis by brain slices.

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