Table 3. *Values of the extracellular volume of lens*

Method	Total space (%)	Readily available space (%)
Chloride analysis and diffusion out into Na_2SO_4	9.6 (sheep) 8.5 (rabbits)	5.5 (sheep) —
Diffusion of ^{24}Na <i>in vivo</i>	—	6.1 (rabbits)
Sucrose (diffusion into lens)	13.5 (sheep)	—
Sucrose (diffusion into lens and subsequent washing in isotonic Na_2SO_4)	—	5.46 (sheep)
Diffusion of ^{82}Br <i>in vivo</i>	—	6.7 (rabbits)

DISCUSSION

For kinetic studies on the penetration of substances from aqueous humour into the lens fibres a knowledge of the magnitude of the effective extracellular space—the ‘readily available space’ discussed here—is a first essential. The results described in this paper indicate that this space is of the order of 6–7 % where *in vivo* studies are concerned. Owing to its greater density, the nucleus of the adult lens contains a higher concentration of potassium (expressed in mg./unit wt. of lens) than the cortex; the ratio $[\text{K}]/[\text{Na}]$ in the nucleus is, however, lower than that in the cortex (Bellows, 1944). If it is assumed that sodium is predominantly an extracellular ion, it might be deduced that the nucleus has a greater extracellular matrix. This conclusion appears doubtful on histological grounds since the hard,

viscous, nucleus is composed of densely packed fibres. A more probable explanation for the lower ratio is that the nuclear fibres are partly permeable to sodium, thereby permitting sodium and chloride to displace potassium, an explanation with which the present findings are in agreement. It is apparent that the nucleus must have a very low metabolism indeed and this fact, coupled with the disappearance of the cell nuclei from these central cells, would support the view of an increased permeability to sodium.

SUMMARY

1. The limitation of the *in vitro* experiment on the excised lens is deduced from studies of the rate at which post-mortem changes occur.

2. Three methods for the determination of the ‘readily available space’ are described.

3. Analysis of the results shows that this space is 5.0–5.5 % of the volume of an adult sheep’s lens and approximately 6 % of the volume of an adult rabbit’s lens.

4. From an analysis of the K/Na ratio in the nucleus and the cortex, together with anatomical and histological evidence, it is suggested that the chloride that fails to diffuse out of the lens within 24–48 hr. is contained in the nucleus.

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Breakdown of Cozymase by a System from Nervous Tissue

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Cozymase has been observed to be unstable in many biological systems (Harden & Young, 1906*a, b*; Schlenk, 1945; McIlwain, 1947; McIlwain & Hughes, 1948). In brain tissue Mann & Quastel (1941) and Handler & Klein (1942) showed the loss of cozymase to be due to a system which liberated nicotinamide from the coenzyme, and which was inhibited by added nicotinamide. Since then many investigators have taken precautions to prevent the breakdown when using systems dependent on cozymase, but the

breakdown has not been studied sufficiently for an assessment to be made of its significance in the normal activities of nervous tissue. Because of the important part played by cozymase itself, we considered it especially necessary to determine the speed of the breakdown, and its variation with cozymase and nicotinamide concentrations and with other factors likely to affect it in the living organism. The breakdown has been found to be very rapid, and to involve the formation of acid (see equation I, p. 474).

EXPERIMENTAL

Materials

Cozymase specimens. Pure cozymase has not been obtained, but the reaction has been carried out with three different specimens of the substance, for which analytical data are given in Table 1. Specimen D was used in the majority of experiments. Specimen A, Table 1, is specimen A of Table 1 of McIlwain & Hughes (1948), and further data concerning it are given in that paper. The determinations of catalytic activity quoted in Table 1 were made in the apozymase system under conditions previously described (McIlwain & Hughes, 1948). The nicotinamide contents quoted for specimens D and E were obtained by liberating the amide by excess of the brain enzyme, and determining the nicotinamide colorimetrically (see below).

Table 1. *Analytical data concerning cozymase specimens*

Determination and standard value	Cozymase specimen		
	A	D	E
I Acid formation in reaction with $\text{Na}_2\text{S}_2\text{O}_4$ (Warburg & Christian, 1936) ($\mu\text{mol./mg.}$)	1.75	2.76	2.58
Purity by comparison with theoretical value of 4.53 $\mu\text{mol./mg.}$ ($\text{C}_{21}\text{H}_{27}\text{O}_{14}\text{N}_7\text{P}_2$; mol. wt. 663) (%)	39	61	57
II Nicotinamide content (%) (theoretical value 18.6)	8.0	11.5	9.9
Purity (%)	43	62	53
III Catalytic activity in apozymase system ($\mu\text{mol. CO}_2/\mu\text{g./hr.}$)	1.47	2.40	—
Purity (by comparison with value of 4.0 derived from Axelrod & Elvehjem, 1939 and McIlwain & Hughes, 1948) (%)	37	60	—
Origin of specimens	This laboratory	Schwartz Laboratories, Inc.	

Tissue preparations. Guinea pigs, rabbits and rats were killed by a blow on the neck, sometimes bled, the skull opened and the brain put on ice, or ground, within 3–4 min. of death. Sheep brain and cord were obtained from a slaughterhouse as soon as possible after killing and brought to the laboratory in ice water. Tissue was ground by hand with an equal weight of acid-washed sand in a cold mortar or homogenized in the glass apparatus of Potter (see Umbreit, Burris & Stauffer, 1945). Crushed tissue was prepared by pressing a fragment approx. $3 \times 3 \times 2$ mm. to approx. 0.3 mm. thickness between slides; it was then handled as a slice. A teased preparation of white matter was made by drawing a fine needle through the material in the direction of the fibres.

Ground preparations with which several experiments were to be performed were prepared as follows (cf. Handler & Klein, 1942). Tissue (25 g.) and sand (25 g.) were rubbed for

5 min. in a mortar and the tissue washed into centrifuge tubes with 100 ml. of 0.9% NaCl, leaving behind the bulk of the sand. After centrifuging, the supernatant was discarded and the precipitate suspended in a further 100 ml. of NaCl. In most instances reported here such washing was performed six times in all, although the preparations did not noticeably change after one or two washings (see p. 472). Preparations were stored at 2–4° as suspensions containing tissue from 1 g. fresh weight of brain in 2 ml. of 0.9% NaCl saturated with toluene. Metabolic quotients were calculated from the dry weights of the whole tissue from which the preparations had been made.

Determination of nicotinic acid and nicotinamide

The reaction with cyanogen bromide (see especially Teeri & Shimer, 1944) has been adapted to the special problems associated with the determination of nicotinamide in the presence of cozymase.

Reagents. The phosphate buffer was that of Martinek, Kirch & Webster (1943). CNBr solution was prepared from sat. Br_2 -water (500 ml.) and KCN (10% w/v, 70–80 ml.) at 5–10° and kept in a refrigerator for not more than a week. The necessary concentration of HCl used was found to be approx. 5N for nicotinic acid and N for nicotinamide.

Nicotinic acid. To the CNBr (2.5 ml.) and buffer (5 ml.) solutions in glass-stoppered tubes, the specimens (containing up to 0.5 $\mu\text{mol.}$ nicotinic acid in 5 ml.) were added and the mixtures left at room temperature for 35 min., *m*-phenylenediamine dihydrochloride (5% in HCl, 0.5 ml.) and HCl (0.5 ml. 5N) were added rapidly, mixing after each addition. The intensities of the resulting yellow colours were determined within 30 min. in a Spekker absorptiometer with Ilford spectrum filter no. 601. A reagent blank (water substituted for the test solution) was subtracted.

Nicotinamide. The amide yielded about a quarter of the colour intensity afforded by the acid under the above conditions (cf. Melnick & Field, 1940; Perlzweig, 1947). Several workers have used heat during the reaction with CNBr to reduce the reaction time and increase the colour intensity (Bandier & Hald, 1939; Martinek *et al.* 1943); in the presence of cozymase we found most satisfactory the following modification of the method described above for nicotinic acid. The mixtures of CNBr, buffer and specimens (up to 0.5 $\mu\text{mol.}$ nicotinamide) were kept at 37° for 30 min., cooled to approx. 10°, the amine (0.5 ml.) and HCl (0.5 ml. N) added, and intensities determined within 40 min.

Preparation of specimens for analysis. Specimens were mainly from reaction mixtures containing brain tissue or preparations, and it was necessary to deproteinize them without liberating nicotinamide from cozymase. Zn(OH)_2 was found satisfactory. To the specimen (containing approx. 5–40 mg. wet wt. of tissue) in a graduated centrifuge tube ZnSO_4 (8% w/v, 0.2–0.4 ml.) was added and the tube put in ice water. NaOH solution, equal in volume to the ZnSO_4 , and of a concentration previously found sufficient to remove the Zn and leave a neutral solution, was added followed by water to 7 or 10 ml. The tubes were centrifuged and samples taken for nicotinamide determination.

Acid formation during cozymase breakdown

In the main compartments of Warburg vessels (conical, of approx. 15 ml.) were placed 2 ml. of a solution containing NaHCO_3 (0.009M) and NaCl (0.9%), freshly gassed with

5% (v/v) $\text{CO}_2\text{-N}_2$. To the side arms were next added 0.5 ml. of the bicarbonate saline (controls) or cozymase freshly dissolved in bicarbonate saline (experimental vessels). The tissue suspension (0.5 ml., in 0.9% NaCl) was then added to the main compartments, yellow P to the centre cups, the vessels fitted to manometers and equilibrated with the $\text{CO}_2\text{-N}_2$ at 37° . Successive readings were usually steady 15–20 min. after placing in the thermostat; the contents were then mixed and readings taken at 3 or 5 min. intervals.

Other physiological salines used were those of Krebs & Eggleston (1940).

RESULTS

Rate of reaction

Estimation by nicotinamide formation. First indications of the high reaction velocity were obtained by following the course of liberation of nicotinamide by brain tissue ground with sand (Fig. 1). Cozymase concentrations were employed which approximated to those of the pyridine nucleotide content of animal tissues. After stopping the reaction and removing protein (both accomplished by zinc hydroxide precipitation) nicotinamide was determined by the Koenig reaction. The initial velocity in Fig. 1 corresponds to the decomposition of $1.8 \mu\text{mol.}$ of cozymase/mg. dry wt. of whole tissue/hr. Other experiments by this method under conditions similar to those of Fig. 1 gave quotients of 1.0–1.4 with tissue from different species (Table 2).

Direct comparison between the rate of breakdown of cozymase and the rate of respiration was made with material from guinea pig brain. Slices (mixed grey and white matter from cerebral hemispheres)

absorbed $0.64, 0.60 \mu\text{mol. O}_2/\text{mg. dry wt./hr.}$ in glucose saline, without Ca salts, at pH 7.4 and at 37° (O_2 as gas phase). Values in saline with Ca (0.005M) were $0.4, 0.38 \mu\text{mol. O}_2/\text{mg. dry wt./hr.}$ These are

Fig. 1. Course of liberation of nicotinamide from cozymase ($0.62 \mu\text{mol.}$) by ground rabbit brain (0.4 mg. dry wt.) in 3.2 ml. phosphate Ringer at pH 7.4; 37° ; aerobic.

typical values (Warburg, 1924; Quastel, 1939; Elliott & Libet, 1942). Ground tissue from the same hemispheres decomposed $1.25, 1.35 \mu\text{mol. cozymase/mg. dry wt./hr.}$

Activity of different tissue preparations. The rate of reaction increased with the degree of damage of the tissue (Table 2), being less in slices or crushed tissue

Table 2. Cozymase breakdown by different species and preparations

Animal	Tissue	Treatment	Rate of breakdown ($\mu\text{mol./mg. dry wt./hr.}$)
Rabbit	Whole brain	Ground with sand	1.4
Guinea pig A	"	"	1.0, 1.4
Sheep A	"	"	1.3
Mouse	"	"	0.5
Sheep B	Cord	Homogenized	1.08
	White matter from corpus callosum	"	0.86
	Grey matter from lateral convexity of hemispheres	"	0.36
	Grey matter adjoining longitudinal fissure	"	0.36
Guinea pig B	Whole cerebral hemisphere	Ground with sand; not washed	1.02
	" "	Ground with sand; washed 3 times	1.03
	" "	Homogenized in equal weight of saline	1.15
	" "	Homogenized in $\times 20$ wt. of saline	1.12
Guinea pig C	Largely white matter from centrum ovalae	Sliced	0.28, 0.46
	Largely grey matter from cerebral cortex	"	0.13
Guinea pig D	Largely corpus callosum	Teased	0.43
	Mixed tissue from cerebral hemispheres	Crushed	0.51
	" " " "	Homogenized	1.56

than in homogenates or material ground with sand. In grinding with sand, little difference was found between a preparation rubbed for 0.5 and one rubbed for 5 min. No appreciable difference was found between the activity of a suspension of guinea pig brain made 2–3 min. after death, and another made after the excised tissue had been left at room temperature for 30 min. White matter was more active than grey (Table 2).

Acid formation during the reaction

The findings of Handler & Klein (1942) on the breakdown of cozymase suggested to us that the end products might be more acidic than cozymase, since the reaction involves the conversion of a pyridinium salt, the salt of a strong base, to a much more weakly basic pyridine derivative (see equation I, p. 474). The breakdown of cozymase has been followed in previous work either by measuring the fall in catalytic activity of cozymase-containing solutions or by measuring the nicotinamide produced. Measurement by acid formation would be much more convenient in studying the reaction kinetically, but it was first necessary to find whether the same breakdown was in fact being observed. Relevant findings are given below.

Fig. 2. Course of evolution of CO_2 from NaHCO_3 -containing solutions during breakdown of cozymase by ground brain (points); relationship to nicotinamide released (crosses). (A) Rabbit; pH, 7.3, maintained by NaHCO_3 (0.021 M) and $\text{CO}_2\text{-N}_2$ (5% v/v). Reaction mixtures contained also 0.9% NaCl, 1.24 $\mu\text{mol.}$ cozymase and the following dry weights of tissue: —○—○—, 1 mg.; —●—●—, 2.5 mg. (B) Sheep; pH 6.8 (by $\text{NaHCO}_3\text{-CO}_2$); cozymase, 2.4 $\mu\text{mol.}$, 4.2 mg. dry weight of tissue; otherwise as (A). The two sets of points (○ and ●) refer to duplicate reaction mixtures. Crosses indicate nicotinamide released at the end of each experiment.

Quantity of acid produced. By carrying out the reaction in bicarbonate-containing solutions in manometric apparatus it was possible to observe a smooth evolution of CO_2 (Fig. 2). The use of such conditions for measuring the rate of the reaction was, therefore,

examined. Anaerobic conditions were generally employed, to minimize the possibility of associated reactions. The brain preparations after three washings with 0.9% sodium chloride caused very little gas change at the low concentrations in which they were used (0.5–2 mg. dry wt./ml.; 3 ml./vessel). Reaction in the absence of cozymase was relatively very small even when fresh homogenates of tissue were used without any washing. The commonest observation was of gas absorption in the absence of substrate, at a rate between 0 and 10% of the rate of reaction with cozymase. Such changes were allowed for by subtracting the change in a tissue control from those in experimental vessels. Any retention of CO_2 by the preparations was within experimental error. Using such methods, the CO_2 evolved was found to be approximately equivalent to the cozymase decomposed (Fig. 2). Seventeen experiments with five different preparations, including sheep, rabbit and guinea pig brain, gave for the ratio: CO_2 evolved/nicotinamide formed, a mean value of 0.98 with standard deviation of 0.08.

With limited quantities of cozymase the gas evolution reached a well-marked end point equivalent to the cozymase added. At this stage the enzyme was not inactive; this was shown by addition of further cozymase. The end point is being approached in one experiment of Fig. 2A.

Inhibition by nicotinamide. Mann & Quastel (1941) found 0.1–0.01 M-nicotinamide prevented the inactivation of cozymase by animal tissues, including brain. This finding has been applied by many subsequent workers in experiments which required

Table 3. *Inhibition of the breakdown of cozymase by nicotinamide*

(Reaction mixtures contained a sheep-brain preparation (1.7 mg. dry wt.) and the nicotinamide in 2.5 ml. NaCl-NaHCO_3 . Cozymase (in 0.5 ml.) was added at zero time, and the reaction run for 26 min. The rates of the last column were determined graphically.)

In reaction mixture		Produced		Initial rate of CO_2 evolution ($\mu\text{mol./mg. dry wt./hr.}$)
Cozymase ($\mu\text{mol.}$)	Nicotinamide ($\mu\text{mol.}$)	Nicotinamide ($\mu\text{mol.}$)	CO_2 ($\mu\text{mol.}$)	
1.24	0	1.05	1.01	1.26
1.24	1.0	0.92	0.96	1.13
1.24	2.0	0.82	0.78	0.75
1.24	4.0	0.60	0.55	0.56
1.24	8.0	—	0.26	0.34

intact cozymase, relatively high concentrations of the amide (e.g. 0.02 M) being employed. We found both 0.01 and 0.002 M-nicotinamide almost completely inhibited the formation of acid from cozymase by washed sheep brain, under the experimental conditions described above. This additional evidence supports the validity of the manometric method for

following cozymase breakdown. Moreover, the reaction was found to be extremely sensitive to nicotinamide. A quantity equimolar to the cozymase content of a typical reaction mixture markedly lowered the reaction rate (Table 3). Because of this sensitivity it was possible to follow the inhibited reaction quantitatively not only by acid formation but also by nicotinamide production. Good agreement was obtained between measurements made in these two ways (Table 3). This makes it probable that the acid and nicotinamide are indeed formed in the same reaction, and, accepting the evidence of Handler & Klein (1942), this may be formulated as in (I), though we have not fully investigated alternative possibilities.

Rate of cozymase breakdown under various conditions

The effect of a variety of circumstances on the breakdown was examined. In doing this the rate of acid formation was largely used, but results were confirmed in each set of experiments by measuring at the end of the reaction the total nicotinamide formed.

pH, salts, oxygen. The reaction showed an optimum pH between 6.5 and 7 (Fig. 3). Most manometric experiments were, therefore, carried out at pH 6.9,

Fig. 3. Change in rate of cozymase breakdown with pH. ●, 0.02M-phosphate buffers, guinea pig brain; O, 0.1M-acetate buffers, guinea pig brain; +, bicarbonate-5% v/v CO_2 , rabbit brain. Initial concentration of cozymase $2 \times 10^{-4}\text{M}$.

obtained by 0.0075M-sodium bicarbonate in equilibrium with 5% (v/v) $\text{CO}_2\text{-N}_2$. Great variation in the ionic composition of the medium was possible without significant change in the reaction velocity

(Table 4 and Fig. 3). The reaction velocity was compared in air/ CO_2 and N_2/CO_2 (with yellow phosphorus to maintain anaerobiosis), and no difference was found.

Table 4. Cozymase breakdown in different media

(All mixtures contained cozymase $(2 \times 10^{-4}\text{M})$ and were at pH 7.4. Each of the velocities quoted was calculated from three determinations of the nicotinamide liberated at different times during the reaction.)

Suspending medium	Rate of breakdown ($\mu\text{mol. nicotinamide}$ liberated/mg. dry wt./hr.)	
	With guinea pig brain	With rabbit brain
Physiological saline with Ca	1.07	1.26
Physiological saline without Ca	1.17	1.25
Phosphate buffer (0.02M)	0.96	1.17

Stability of preparations. Ground sheep brain, washed and suspended (4.4 mg. dry wt./ml.) in 0.9% sodium chloride at pH 7.0, retained less than 5% of its activity after heating for 15 min. at 100 or 70°. At 60°, 20% of the activity remained and at 50°, 40%. At 37°, no loss occurred in 1 hr. at pH values (in phosphate) of 5.6, 6.8 and 8. Preparations were normally stored in a refrigerator at 2–3°, with 0.5 g. wet wt. of tissue/ml. of 0.9% sodium chloride. Most preparations had been washed with 0.9% sodium chloride (volume, five times the bulk of fresh tissue) by centrifuging either three or six times, and were, without deliberate adjustment of pH, at pH 7. Toluene was added as a preservative and under these conditions little loss in cozymase-splitting activity occurred during 1–2 months. Some loss was found in preparations 3 or 4 months old, and in a more recent preparation in which bacteria had grown.

Variation in velocity with cozymase concentration; different cozymase specimens. Figs. 1 and 2 show that the velocity of breakdown of cozymase falls towards the end of experiments in which cozymase concentration is decreasing, and the concentration of nicotinamide, which inhibits the reaction, is increasing. To differentiate between these two factors, kinetic experiments were carried out with different initial concentrations of cozymase, and initial velocities of breakdown obtained from graphs such as those of Fig. 2. The initial velocity fell relatively little at concentrations down to $6 \times 10^{-4}\text{M}$, the lowest at which the ordinary manometric method is applicable. The reaction velocity at concentrations below this was, therefore, followed by nicotinamide determinations. The results (Fig. 4) were not sufficiently accurate for determining the equilibrium constants of the system, but showed that the velocity did not fall to half the maximum until the concentration of cozymase was less than 10^{-4}M . A higher value is given by Spaulding & Graham (1947) for unspecified

tissues which may include brain. Rates of breakdown of specimens A, D and E of cozymase (see Table 1) were compared at concentrations between 1.3 and $4 \times 10^{-4} M$; no differences were found.

Fig. 4. Change in initial rate of cozymase breakdown with cozymase concentration, in two preparations of guinea pig brain. Above: cozymase E; below, cozymase D. ○, determined by CO_2 evolution; ●, by nicotineamide formation.

Extraction of cozymase-splitting enzyme

In the preceding experiments a suspension of tissue particles in 0.9% sodium chloride was used in which the activity was firmly associated with the particles (Table 2). The active material readily dissolves in water with little loss, together with a large part of the tissue preparation, giving an opalescent solution; as is shown in Table 5 the activity/unit dry weight is increased in the process. Sheep brain has been extracted similarly, both before and after standing with toluene. The properties and treatment of such extracts will be described later.

DISCUSSION

The following findings must be taken into account in assessing the part played by the breakdown of cozymase in preparations from nervous tissue. (1) Its velocity of 0.4 – $1.8 \mu\text{mol./mg. dry wt./hr.}$ This is outstanding. The rate of reaction, even on a molar

basis, can be greater than the respiratory rate of the tissue; it can lead to the breakdown of the tissue's own dry weight of cozymase each hour. (2) This velocity is reached at the concentrations of cozymase occurring in the tissue. (3) The reaction is inhibited by nicotineamide, also at concentrations comparable to those at which nicotineamide derivatives occur in the tissue. It is (4) most rapid in damaged tissue, leads (5) to loss of the catalytic activity of cozymase and (6) to the formation of hydrogen ions at an equivalent, high velocity.

Table 5. *Extraction of cozymase-splitting system from guinea pig brain*

Preparation	Activity (by CO_2 evolution at pH 6.8)	
	$\mu\text{mol. cozy-mase split/material derived from 1 g. fresh wt./hr.}$	$\mu\text{mol. cozy-mase split/mg. dry wt. of preparation/hr.}$
(1) Whole brain ground with sand	224	1.12
(2) Preparation (1) washed with saline and extracted with water (5 ml./g. fresh brain); extract	77	1.6
(3) Residue from (2) re-extracted as before; extract	77	1.4
(4) Residue from (3)	58	0.6
Sum of (2), (3) and (4)	212	—

Reaction velocity. Although the reaction has been investigated in ground nervous tissue and has frequently been described as rapid, kinetic experiments leading to dependable values for its rate do not appear to have been reported. An isolated statement is made by Spaulding & Graham (1947, in a footnote to Table 1), but not discussed further, that 'the nucleosidase/g. of dry brain...split approximately 15 mm of DPN in 20 min.' Interpreting mm as millimol., the velocity corresponds to 100 times that which we find; on the other hand, most other measurements of cozymase in Spaulding & Graham's paper are in micromol. quantities, and if mm means micromol. the speeds are about 1/10 those which we find. Spaulding & Graham give the relative velocities of breakdown by ground rat brain and heart as in the ratio 100:27. The rate of breakdown of cozymase by guinea pig heart can be derived indirectly from measurements by Govier & Jetter (1948) as about $0.3 \mu\text{mol./mg. dry wt./hr.}$; ignoring possible species differences, this would suggest a value of about $1.1 \mu\text{mol./mg./hr.}$ for brain. This is close to our own findings and appears to be the most satisfactory interpretation of the observations of Spaulding & Graham (1947). We have preferred not to use a name

such as diphosphopyridine nucleotidase for the system, as the specificity of its action has not yet been examined.

Dependence on nicotinamide and cozymase concentrations. The concentration of cozymase in fresh brain tissue can be calculated from values given by Axelrod, Madden & Elvehjem (1939) as approx. 5×10^{-4} M. Values for total nicotinic acid derivatives are close to this (Mitchell & Isbell, 1942; Taylor, Pollack & Williams, 1942). With such an initial concentration of cozymase, our results show that the breakdown can proceed at over 80 % of its maximum velocity, but that a fall in cozymase concentration results in a fall in the velocity of the reaction (Fig. 4). Also, the reaction is inhibited by comparable concentrations of nicotinamide, one of its products. Therefore, although the operation of this reaction at its maximum velocity would lead to very rapid depletion of the cozymase of the tissue, the velocity of breakdown would be expected to fall considerably as the substrate disappeared and the product accumulated.

Possible operation of the reaction in vivo. Although the breakdown of cozymase is particularly rapid in brain tissue, and may there subserve a special purpose, it occurs in many animal tissues (cf. Schlenk, 1945; Spaulding & Graham, 1947; Govier & Jetter, 1948) and also in bacteria (McIlwain & Hughes, 1948). Data are only rarely available for full characterization of the reaction, but it is readily differentiated from that of the nucleotide pyrophosphatase (Kornberg, 1948) which does not liberate free nicotinamide. The process in streptococci liberated nicotinamide, but was not associated with acid formation (McIlwain & Hughes, 1948); there, however, intact cells were being examined, and greater possibility existed for coupling with further changes. The major problem in assessing the physiological importance of the reaction lies in reconciling its high velocity with the undoubted importance of intact cozymase. In general, this suggests that the breakdown may be intermittent, owing to some physiologically controlled inhibition; that the breakdown must be counterbalanced by synthesis; and that the reaction may constitute part of a system controlling cozymase-requiring reactions by regulating the cozymase level.

Considering more specifically mammalian brain, the cozymase content of the fresh organ in a given species has been found to be relatively constant (Axelrod *et al.* 1939), and breakdown of added cozymase is smallest when least damage has been done to the tissue. In the tissue, which of course contains more than one cell type, the process is presumably inhibited for a large part of the time (possibly by

separation of the reactants), and balanced by re-synthesis if and when it takes place. Stability of the native cozymase of the tissue is being examined separately, but it is possible to make the following observations from the present data. The reaction seems suited to physiological operation and control in that its pH optimum is within physiological range; its relation to substrate concentration is such that with the cozymase concentration found in the tissue as a whole the reaction would be rapid initially, but decrease in rate as cozymase is lost and nicotinamide accumulates; the high initial velocity makes it less likely that reaction with cozymase is an incidental property of a system whose main reaction is with another substrate. The phenomena of functional importance in nervous tissue are intermittent, extremely rapid and associated with ion migration and permeability changes. These give many possibilities for the functional importance of a reaction with the characteristics of the cozymase breakdown, but more data are required for their detailed discussion.

SUMMARY

1. The process by which tissue preparations from the central nervous system liberate nicotinamide from cozymase proceeded at rates of 0.4–1.8 $\mu\text{mol./mg. dry wt./hr.}$; this was often greater than the rate of respiration by the tissue.
2. The reaction approached its maximum velocity at the natural concentration of cozymase in brain tissue (5×10^{-4} M); half the maximum velocity was reached at less than 10^{-4} M-cozymase.
3. The breakdown was very sensitive to nicotinamide; 10^{-3} M-nicotinamide reduced the rate of breakdown of 3×10^{-4} M-cozymase to half its uninhibited value.
4. The process resulted in the formation of one equivalent of acid/molecule of nicotinamide liberated. This was apparently due to the disappearance of the pyridinium ion, and when the reaction was carried out in $\text{NaHCO}_3\text{-CO}_2$ buffers its velocity could readily be followed manometrically by CO_2 evolution.
5. The optimum pH of the reaction was approx. 7, and the velocity was almost unaffected by many inorganic salts.
6. The system responsible for the reaction remained associated with tissue debris in isotonic solutions, and was fairly stable at 0 or 37°, but inactivated rapidly at 70°. It was easily obtained in solution in water.

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The Oxidation of Catechol and Homocatechol by Tyrosinase in the Presence of Amino-acids

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In the early stages of the oxidation of catechol by crude extracts of potato or mushroom the formation of reddish purple pigment can be detected. Attention was drawn to this phenomenon first by Szent-Györgyi (1925), who achieved some degree of separation from potato juice of the substance which, together with enzyme and catechol, was responsible for the pigment formation. He suggested the name 'tyrin' for it and speculated as to its possible significance as a reversibly oxidizable hydrogen carrier. Platt & Wormall (1927), however, subsequently concluded that 'tyrin' was merely a mixture of amino-acids, and no more has been heard of it. Although, as Platt & Wormall believed, many amino-acids can cause pigment formation when present in solutions in which tyrosinase is oxidizing catechol, there is now reason to think that the amino-acid contributing most strikingly to the phenomenon originally observed is L-proline. The identification of L-proline as a constituent of mushroom extract, and the results of a study of some features of the reaction involved in the production of the pigment, are described in this paper.

METHODS

Tyrosinase preparations. These were made from the common mushroom (*Psalliota campestris*) according to the directions of Keilin & Mann (1938), but with the omission

of some of the later steps in the purification described by these workers. Various preparations with Q_{O_2} values ranging between 50,000 and 500,000,* were used in the course of the work. Within these limits variation in the purity of the enzyme preparation has no qualitative effect on the reactions which are to be discussed.

Measurement of oxygen uptake. The Warburg manometric apparatus was used. The total volume of reaction mixture was 2.0 ml., the components being distributed in 0.1M-phosphate buffer of pH 7.0. The enzyme was tipped into the substrate solution from the side arm at zero time, and in critical experiments the inset cup of the reaction vessel contained 20% KOH and a roll of filter paper. In the earliest experiments the temperature of the water bath was 20°, but was changed to 25° when the advent of warmer weather made the maintenance of the lower temperature difficult.

Colorimetry. In experiments where quantitative assessment of pigment formation was attempted a Spekker photo-electric absorptiometer was used with a 1 cm. cell and a green filter (no. 5 of Hilger set H 455, later replaced by Ilford spectrum green no. 604). The destruction of the pigment in acid solution was similarly studied, readings being taken at timed intervals after addition of acid to the pigment solution.

Measurement of pH and hydrogen-ion liberation. A glass electrode was used for pH measurement. When it was desired to study the hydrogen-ion liberation accompanying the oxidation of catechol in the presence of amino-acid the reaction was carried out in unbuffered solution at pH 7.0.

* Q_{O_2} values in $\mu\text{l./mg. dry wt./hr.}$, substrate catechol, determined manometrically according to Keilin & Mann (1938).