Ammonia Formation in Brain

1. STUDIES ON SLICES AND SUSPENSIONS

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It has been known for some time that brain tissue is capable of forming considerable amounts of ammonium ions *in vivo* and *in vitro* (for review of literature see Weil-Malherbe, 1950, 1953*b*), but its origin and the mechanism of its formation are still obscure. The experiments reported in this paper were undertaken to study these problems further.

METHODS

Tissue preparations and conditions of incubation

Slices of brain grey matter were cut freehand with a moist single-edged safety-razor blade and stored in ice-cold bicarbonate-Ringer solution. Batches of 60–80 mg. (wet wt.) were used in 3 ml. suspension medium. A similar batch was used for the estimation of the wet wt./dry wt. ratio. Wet weights were corrected for swelling by multiplying the calculated dry wt. with an assumed wet wt./dry wt. ratio of 5.0.

Whole rat brains were used for the preparation of suspensions. The tissue was ground in a Potter-Elvehjem homogenizer in 1 vol. of medium and diluted to the required strength.

Brain slices were suspended in bicarbonate-Ringer solution (Krebs & Henseleit, 1932). The following media were used in experiments with brain suspensions: (1) a solution of 0.020M-NaHCO₃ ('water suspension'); (2) an alkaline potassium-rich salt solution (modified from Potter, 1948), containing KCl 0.05M, KHCO₃ 0.05M, potassium phosphate buffer (pH 7.6) 0.01M; and (3) an isotonic saline (modified from Birmingham & Elliott, 1951), containing NaCl 0.12M, NaHCO₃ 0.025M, KCl 0.003M, KH₂PO₄ 0.0004M, MgSO₄ 0.0012M. The final concentration of the brain suspensions was 1 in 30.

In some experiments brain suspensions were used after dialysis against running isotonic salt medium for 3 hr. at 3° or after three washings of the centrifuged residue with the same medium.

Rat-brain mitochondria were collected as the fraction thrown down between 1200 and 15000 *g* in isotonic sucrose. The residue was resuspended 3 times in sucrose and incubated in a medium containing KCl 0.1M, MgSO₄ 0.01M, and potassium phosphate buffer (pH 7.4) 0.05M.

Unless specifically denoted as anaerobic, all incubations were carried out in an atmosphere of 5% (v/v) CO₂ in O₂. A gas mixture of 5% (v/v) CO₂ in N₂, freed from oxygen by passage over red-hot copper wire, was used in anaerobic experiments. The flasks were shaken in a water bath at 37°.

Analytical

General. In experiments with brain slices 0.1 vol. 50% (w/v) trichloroacetic acid (TCA) was added at the end of the incubation period or, where initial values were required, before the addition of the slices. The tissue was thoroughly broken up with a glass rod and the mixture filtered through Whatman no. 5 paper. The whole of the filtrate or a suitable portion was used for the estimation of free ammonia and amide N. When other fractions were to be determined, the mixture of slices, medium and TCA was rinsed into a graduated centrifuge tube with 5% (w/v) TCA, some acid-washed sand added and, after thorough grinding, the volume made up to 6 ml. with 5% TCA. After quickly centrifuging, the supernatant was filtered through Whatman no. 5 paper. Portions of 0.5–1 ml. of filtrate were used for the estimations of acid-soluble P and non-protein N (NPN) and the remainder was used for the estimation of free ammonia and amide N.

In experiments with brain suspensions, 2-ml. samples were withdrawn with a wide-tipped pipette and delivered into 2 ml. 12% (w/v) TCA. When initial values were to be determined, this was done immediately after the addition of the brain suspension. Of the filtrate, 2 ml. were used for the estimation of free ammonia and amide N and 1 ml. for the estimation of NPN.

Estimation of ammonia and amide N. These two estimations were carried out in the same solution by the method of Krebs (1935). A distillate of 10 ml. was collected in the apparatus of Parnas & Heller (1924). When only free ammonia was estimated, Conway units were used with 2 ml. filtrate and 1 ml. saturated K₂CO₃ in the outer ring (cf. Conway, 1950), followed by nesslerization of the solution contained in the centre compartment (see below).

Estimation of total nitrogen. Micro-Kjeldahl determinations were carried out by the Conway microdiffusion method after digestion with a mercury catalyst. Since the use of a mercury catalyst has been considered inconvenient or unsuitable in conjunction with the microdiffusion technique (Conway, 1950), particulars of the procedure are given. To the sample containing 5–50 µg. N is added 1 ml. of a solution containing 0.625% (w/v) HgO in 9N-H₂SO₄. After the water has been boiled off, 100 mg. K₂SO₄ are added. Digestion is continued for 2 hr. after clearing. The cooled digestion mixture is diluted to 5 ml. and a sample of 2 ml. is placed in the outer chamber of a Conway unit, followed by 1 ml. of a solution containing 10% (w/v) Na₂S₂O₃·5H₂O in 60% (w/v) KOH. The inner chamber contains 1 ml. 0.01N-HCl. When the diffusion of ammonia is complete the contents of the inner chamber are removed with a fine-tipped test pipette and transferred to a test

tube, followed by two washings with 1 and 0.5 ml. water respectively; 0.25 ml. Nessler reagent is then added. Readings are taken in colorimeter cells with capacity 3.5 ml. and diam. 1 cm.

The method gave theoretical recoveries with various purines and amino acids.

Phosphate fractions. After a further washing in 5% TCA the insoluble tissue residue was fractionated by the method of Schmidt & Thannhauser (1945) as modified by LePage & Heidelberger (1951). The total N was estimated in a sample of the alkaline digest and used for the calculation of 'protein N' as described by Leslie & Davidson (1951). Total P (5–10 $\mu\text{g.}/\text{sample}$), after digestion with 60% (w/w) perchloric acid (King, 1932), was estimated as previously described (Weil-Malherbe & Green, 1951; Weil-Malherbe, 1953c).

Materials

Diphosphopyridine nucleotide. This was prepared according to LePage (1947). The substance was standardized spectrophotometrically according to Kornberg (1950); it was 48.5% pure.

Inosine triphosphate (ITP). This was prepared according to Lehninger (1945). The Ba salt had: inorganic P, 0.16; organic P, 10.3; acid-labile P, 6.61; N, 6.44%. Calc. for $\text{C}_{10}\text{H}_{11}\text{O}_{14}\text{N}_4\text{P}_3\text{Ba}_2 \cdot 7\text{H}_2\text{O}$: organic P, 10.3; acid-labile P, 6.86; N, 6.19%.

Creatine phosphate. This was prepared according to Zeile & Fawaz (1938) and converted into the barium salt. (Found: inorganic P, 0.29; organic P, 8.76; acid-labile P, 8.78%. Calc. for $\text{C}_4\text{H}_8\text{O}_5\text{N}_3\text{PBa}$: P 8.95%.)

D-Glutamic acid. This was a gift from Professor H. A. Krebs. For analytical data see Krebs (1935).

Cytochrome c. Landrax (Evans Medical Supplies Ltd., Liverpool and London) was dialysed until free of ammonium ion.

Other materials. These were of commercial origin and were used without further purification.

RESULTS

Experiments with brain slices

Ammonia formation. It has previously been shown (Weil-Malherbe, 1936) that brain slices respiring in a glucose-free medium form ammonia at a fairly steady rate for several hours. This observation has now been confirmed by a larger number of experiments (Fig. 1). In most experiments slices of guinea-pig brain were used, but very similar results were also obtained with slices of rat brain. The curve of ammonia formation is slightly S-shaped with its steepest part in the period 0.5–2 hr.

The rate of ammonia formation is strongly and progressively inhibited under anaerobic conditions as well as aerobically in the presence of mM 2:4-dinitrophenol (DNP). In Ringer solution containing 0.2% glucose the ammonia formation is completely suppressed during the first 2 hr. and slightly increases thereafter. The inhibition by glucose and other substrates of the ammonia formation by brain slices has previously been observed by

Warburg, Posener & Negelein (1924), Loebel (1925), Dickens & Greville (1933) and Weil-Malherbe (1936).

Amide nitrogen. When guinea-pig brain slices are respiring in a substrate-free medium, the amide concentration decreases during the first hour (Fig. 2). This change is probably significant ($P=0.04$), but the subsequent increase found after 2 hr. is not ($P=0.09$); neither is the difference between the initial and the final values ($P=0.16$). Under these conditions, therefore, the amide concentration appears to remain more or less constant

Fig. 1. Ammonia formation of guinea-pig brain slices. Curve 1 (●—●), in glucose-free Ringer soln. (means of 13 expts. with s.e.); curve 2 (×—×), in glucose-Ringer soln. (means of 6 expts. with s.e.); curve 3 (○—○), in glucose-free Ringer soln., anaerobic (means of 4 expts. with s.e.); curve 4 (Δ—Δ), in Ringer soln. containing 10^{-3}M 2:4-dinitrophenol.

Fig. 2. Concentration of amide N in guinea-pig brain slices. Curve 1 (●—●), in glucose-free Ringer soln. (means of 9 expts. with s.e.); curve 2 (×—×), in glucose-Ringer soln. (means of 5 expts. with s.e.); curve 3 (○—○), in glucose-free Ringer soln., anaerobic (means of 3 expts.).

for several hours. It is also stable under anaerobic conditions. In the presence of glucose, on the other hand, a steady and significant rise of the amide concentration is found in aerobic experiments.

Effect of inhibitors on ammonia formation. In addition to DNP, the effect of other inhibitors on the ammonia formation of brain slices was studied

ammonia formation occurring without added substrate (Table 2).

Sulphanilamide caused a slight increase of ammonia formation, which is probably significant since it was observed on three occasions. The possibility that sulphanilamide was itself deaminated cannot be excluded, however.

Table 1. *Ammonia formation of guinea-pig brain slices in the presence of various substances*

Addition	Incubation, 4 hr.		Change or mean change (%)
	Concentration (M)	No. of expts.	
Hydrazine	10^{-2}	3	-67.4
Octan-2-ol	Saturated soln.	3	-66.0
Arsenite	10^{-3}	3	-57.3
Cyanide	10^{-3}	1	-42.5
Iodoacetate	10^{-3}	1	-42.4
Hydroxylamine	10^{-3}	1	-42.0
Sodium fluoride	10^{-2}	1	-38.3
Sodium sulphide	10^{-3}	1	-35.0
Methylene blue	0.05% (w/v)	1	-28.0
Sodium azide	10^{-3}	1	-26.6
Urethane	10^{-2}	3	-25.5
Fluoroacetate	0.5×10^{-3}	3	-8.6
D,L-Methionine sulphoxides (mixture of four isomers)	10^{-2}	2	-5.5
Ephedrine	10^{-2}	Time curve with 5 points	± 0
Sulphanilamide	0.5×10^{-3}	3	+24.1

Table 2. *Effect of glutamine and D-glutamic acid (both at 0.002 M) on the ammonia formation of guinea-pig brain slices*

Incubation, 3 hr.	
Addition	NH ₃ formed (μ moles/100 mg. tissue)
None	1.62
L-Glutamine	6.65
D-Glutamic acid	1.65
L-Glutamine + D-glutamic acid	2.07

(Table 1). Significant inhibitions were produced by those that are known to interfere with electron transport or oxidative phosphorylation. On the other hand, some that might have been expected to influence ammonia formation were found to have no effect. Fluoroacetate, for instance, increases the formation of ammonia in brain *in vivo* (Benitez, Pscheidt & Stone, 1954), but does not do so *in vitro*. Ephedrine is an inhibitor of amine oxidase (Blaschko, Richter & Schlossmann, 1937; Mann & Quastel, 1940), but does not affect the ammonia formation of brain slices. The same is true for methionine sulphoxide, which inhibits the synthesis of glutamine (Borek, Miller, Sheiness & Waelisch, 1946). D-Glutamic acid is a potent inhibitor of glutaminase (Krebs, 1935) and virtually abolishes the ammonia formation from added glutamine by brain slices, yet it does not inhibit the

Fig. 3. Concentration of non-protein N in guinea-pig brain slices. Curve 1 (●—●), in glucose-free Ringer soln. (initial and final values are the means of 16 expts., intermediate values the means of 6 expts., with s.e.); curve 2 (×—×), in glucose-Ringer soln. (initial and final values are the means of 13 expts., intermediate values the means of 6 expts., with s.e.).

In addition to those mentioned in Table 1 the following substances were tested, but they had no significant effect on the ammonia formation of brain slices: arsenate, oxalate, malonate, maleate, malononitrile, acetonitrile, benzaldehyde (all at 0.01 M); creatine phosphate, ITP, inosinic acid (at 0.0025 M); Congo red, pyocyanine, porphyrindin (at 0.05 %); *p*-chloromercuribenzoate, benzylpenicillin, *o*-phenanthroline (at 0.0005 M).

Non-protein nitrogen. As shown in Fig. 3 there is a significant increase of NPN during a 5 hr. incubation of brain slices, both in the presence and in the absence of glucose ($P<0.01$), but the effect is greater in the glucose-free medium. The difference between the two media is probably significant after 5 hr. ($P=0.05$), though not for the earlier values, but this may be due to the smaller number of samples.

Fig. 4 shows the changes of the three nitrogen fractions in brain slices incubated with and without glucose. The increase of ammonia in the absence of

glucose accounts for a larger proportion of the newly formed NPN than that which is accounted for by the sum of ammonia and amide N in the presence of glucose.

Acid-soluble phosphorus. The changes of acid-soluble phosphorus during the first 2 hr. of incubation are slight (Fig. 5). In both media an initial decrease is observed, which is, however, not significant. Later there is a rise which, after 5 hr., reaches a value significantly higher in the glucose-free than in the glucose-containing medium ($P<0.01$). Although, in the absence of glucose, the increase after 5 hr. is significant whether compared with the initial or the 30 min. value ($P<0.01$), in the presence of glucose only the difference between the 30 min. and the 5 hr. values is significant ($P=0.02$).

Lipid and nucleic acid phosphorus. After the incubation of brain slices in a glucose-free medium for 5 hr. a small, but probably significant, decrease occurs in the lipid-P fraction (see also Sperry, 1947) and a relatively larger and highly significant decrease in the ribonucleic acid (RNA) fraction (Table 3). With glucose present the changes are

Fig. 4. Changes of non-protein N, ammonia and amide N during incubation of guinea-pig brain slices, in the presence and absence of glucose. Non-protein N, whole column, measured from 0; ammonia, shaded part of column; amide N, cross-hatched areas.

Fig. 5. Acid-soluble phosphorus in guinea-pig brain slices. Curve 1 (●—●), without glucose (initial and final values are means of 13 expts., intermediate values means of 5 expts., with s.e.); curve 2 (×—×), with glucose (initial and final values are means of 12 expts., intermediate values means of 5 expts., with s.e.).

Table 3. Changes in lipid, nucleic acid and protein fractions of guinea-pig brain slices during an incubation of 5 hr. in the presence and absence of glucose (0.2 %)

		No. of observations	μmoles/100 mg. tissue			P (probability factor)
	Glucose		Initial*	Final*	Difference	
Lipid P	Absent	9	6.45 ± 0.156	6.08 ± 0.081	- 0.37	0.05
	Present	7	6.21 ± 0.105	5.86 ± 0.151	- 0.35	0.08
RNA P	Absent	9	1.13 ± 0.020	0.858 ± 0.0086	- 0.27	< 0.01
	Present	7	1.17 ± 0.021	1.08 ± 0.043	- 0.09	0.07
DNA P	Absent	9	0.223 ± 0.0227	0.180 ± 0.0106	- 0.04	0.12
	Present	7	0.214 ± 0.0086	0.210 ± 0.0118	- 0.004	> 0.2
Protein N	Absent	8	91.3 ± 3.68	80.4 ± 3.55	- 10.9	0.05
	Present	7	89.8 ± 2.96	82.5 ± 3.59	- 7.3	0.15

* Mean with s.e.

smaller and no longer significant. The deoxy-ribonucleic acid (DNA) fraction remains unchanged in both media. Admittedly the so-called RNA fraction includes other phosphorylated compounds in addition to RNA, especially when the analysis is done on brain tissue (Logan, Mannell & Rossiter, 1952). Since only the acid-soluble P was estimated in the acidified alkaline digest, the fraction also includes phosphoproteins.

The last row of Table 3 contains figures for the protein N, calculated according to Leslie & Davidson (1951). The method consists in estimating the nitrogen content of the alkaline digest before acidification. 'Protein N' is obtained by subtracting the amount of N corresponding to the sum of RNA-P and DNA-P, the assumption being made that 1 g. nucleic acid P corresponds to 1.69 g. nucleic acid N. Although there are obvious objections to this procedure, mainly on account of the low specificity of the RNA estimation, the corrections are relatively small and are, in fact, of the same order of magnitude as the standard error of the mean of the protein N values. After an incubation of 5 hr. a decrease of the protein N is found with and without glucose, but this is only significant without glucose. Though small in relation to the size of the protein N, the decrease is large enough to account amply for the increase of NPN.

Experiments with brain suspensions

When rat brain is dispersed in a hypotonic NaHCO_3 buffer, the initial ammonia content is about 1.0–1.5 $\mu\text{moles}/100 \text{ mg. tissue}$; it increases only slightly during subsequent incubation. When isotonic salt solution is used, the initial ammonia concentration is lower and subsequent aerobic incubation causes a significant increase. Under anaerobic conditions the curve is similar to that found with the 'water suspension' (Fig. 6). The results on the mean ammonia formation of guinea-pig brain slices have been included in Fig. 6 for comparison.

DNP has an inhibitory effect on the ammonia formation of a brain suspension in isotonic salt solution, but this is less pronounced than in the slice. On the assumption that the ammonia formation depends on oxidative phosphorylation, the effect of adding high-energy phosphate groups in the form of ITP was investigated. ITP was chosen rather than ATP since it does not liberate ammonia in contact with tissues; it is an effective phosphate donor owing to the presence of nucleoside diphosphokinase in tissues (Krebs & Hems, 1953; Berg & Joklik, 1954). ITP, far from increasing the ammonia formation of the isotonic suspension, inhibited it to an extent similar to the effect of DNP. No summation of effects occurred when the two substances were added together (Table 4).

The differences between the results obtained with slices and with 'isotonic' suspensions may to some extent be due to the larger admixture of white matter in the latter, but if this factor entirely accounted for them one would expect a closer qualitative resemblance of the two time curves (Fig. 6, nos. 1 and 4). In addition to those already

Fig. 6. Ammonia formation of brain preparations. Curve 1 ($\times-\times$), rat-brain dispersion in isotonic medium, aerobic (means of 5 expts. with s.e.); curve 2 ($\text{O}-\text{O}$), rat-brain dispersion in hypotonic medium (means of 2 expts.); curve 3 ($\Delta-\Delta$), rat-brain dispersion in isotonic medium, anaerobic (means of 3 expts.); curve 4 ($\bullet-\bullet$), guinea-pig brain slices (means of 13 expts.).

Table 4. Effect of 2:4-dinitrophenol (10^{-3} M) and inosine triphosphate ($2.5 \times 10^{-3} \text{ M}$) on the ammonia formation of an isotonic suspension of rat brain

Addition	NH ₃ formed ($\mu\text{moles}/100 \text{ mg. tissue}$)			
	0	1 hr.	2 hr.	4 hr.
None	1.41	2.05	2.25	2.47
ITP	1.41	1.66	2.12	2.19
DNP	1.41	1.75	2.10	2.10
ITP + DNP	1.53	1.80	1.97	2.04

mentioned, other attempts were made to influence the ammonia formation of brain dispersions, but the following additions, singly or combined, were without effect: MgCl_2 (0.005 M), nicotinamide (0.04 M), diphosphopyridine nucleotide ($6 \mu\text{g./ml.}$),

NaF (0.02M), cytochrome *c* (0.5×10^{-4} M). The basal medium in these experiments was the potassium-rich salt solution.

The formation of NPN during the incubation of brain dispersions was studied in isotonic medium under aerobic and anaerobic conditions (5 and 2 expts., respectively) and in hypotonic medium under aerobic conditions (2 expts.). The experiments lasted for 5 hr. and intermediate values were determined after 0.5, 1, 2 and 3 hr. No significant changes were observed, all values lying in the range 10.8–13.0 μ moles/100 mg. tissue.

Experiments with washed or dialysed preparations

Many of the components that make up the NPN fraction of brain, including 15 L-amino acids (alanine, arginine, aspartate, cysteine, glutamate, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine and valine, all at 0.01M; cystine, saturated soln.; glutathione (–SH), 0.01M), were tested for their ability to form ammonia in experiments with dialysed or washed brain suspensions. A significant ammonia formation was obtained only from the following: ATP, ADP, adenylic acid, adenosine, L-glutamine and L-glutamic acid. This agrees with what is known of the deaminating systems occurring in brain. Various combinations of substrates, especially amino acids, were also tried, but except for the combination aspartic acid–glutamic acid no significant effects were found. Aspartic acid slightly increased the ammonia formation from glutamic acid, an effect which can be explained by the transamination reaction. Since these experiments did not contribute anything to our understanding of the ammonia formation of brain slices they are not reported in detail.

It was observed during these experiments that the addition of ITP greatly increased the ammonia formation from glutamine. A combination of inosinic acid and creatine phosphate had the same effect, whereas either of these substances alone had little or no activity. This was at first interpreted as indicating a reamination of inosine phosphate or polyphosphate by glutamine and subsequent deamination by adenylic deaminase (Weil-Malherbe, 1953a). It was later found, however, that the effect disappeared when a phosphate buffer was substituted for the bicarbonate buffer previously used. Presumably the effect was due to the liberation of phosphate from ITP and to the well-known stimulation of glutaminase by inorganic phosphate (Errera & Greenstein, 1949).

It was further found that washed rat-brain mitochondria possessed high glutaminase and adenylic deaminase activities.

DISCUSSION

These results show that the ammonia formation of brain tissue *in vitro* has two components, one that is presumably mainly autolytic, while the other depends on the structural and functional integrity of the cell or its elements. The structural integrity of subcellular elements is probably better preserved in the isotonic suspension than in the 'water suspension', though of course not so well as in the slice. The ammonia formation of the isotonic suspension is therefore intermediate between those of the other two preparations. The functional integrity of the cell is impaired by anoxia, respiratory inhibitors or 'uncoupling' agents; under these conditions the ammonia formation of slices and isotonic suspensions is lowered to the level observed in water suspensions. Although the ammonia content of brain *in vivo* is only about 0.02–0.03 μ mole/100 mg. (Schwarz & Dibold, 1932; Richter & Dawson, 1948) the initial value found in water suspensions was about 1.5 μ moles/100 mg. In slices the initial value was about 0.5 μ mole/100 mg., although the time interval since the death of the animal was longer than in the experiments with suspensions. This suggests that in the slice, and to some extent in the isotonic suspension, the autolytic processes are inhibited.

The following five deaminating enzyme systems are known to occur in brain: glutaminase, glutamic dehydrogenase, amine oxidase, adenylic deaminase and adenosine deaminase. These five enzyme systems and their possible implication in the ammonia formation of brain slices in a substrateless medium will now be considered.

Glutaminase. It has previously been shown (Weil-Malherbe, 1936) and confirmed in the present investigation that the amide concentration remains fairly constant in brain slices, even under anaerobic conditions. In any case the amount of preformed amide N could account for only a small proportion of the ammonia evolved. The fact that the ammonia formation of brain slices is unaffected by methionine sulphoxide as well as by D-glutamic acid, one of which inhibits glutamine synthesis and the other glutamine hydrolysis, seems to exclude the possibility of a cycle of glutamine synthesis and deamination.

Glutamic dehydrogenase. It is known (Krebs, 1935; Weil-Malherbe, 1936) that during the oxidation of added L-glutamic acid by brain slices no free ammonia arises, owing to the simultaneous synthesis of glutamine according to the reaction:

The addition of oxoglutaric acid results in an inhibition of ammonia formation and, if glucose be

present, even in the disappearance of added ammonium ion (Weil-Malherbe, 1936). This is due to the fact that the equilibrium of the glutamic dehydrogenase reaction is strongly in favour of the reductive amination of oxoglutaric acid (Euler, Adler, Günther & Das, 1938; Strecker, 1953). It is also unlikely that a cycle of transamination, with oxoglutaric acid as amino acceptor, is involved in the ammonia formation of brain slices, since in that case the addition of oxoglutaric acid might be expected to increase rather than decrease the evolution of ammonia. Finally, the fact that the ammonia formation of isotonic suspensions was unaffected by the addition of nicotinamide or of nicotinamide + diphosphopyridine nucleotide also argues against the involvement of glutamic dehydrogenase, in view of the rapid destruction of preformed diphosphopyridine nucleotide in such a preparation (McIlwain & Rodnight, 1949).

Amine oxidase. A survey of the nitrogenous components of deproteinized brain extracts (Weil-Malherbe, 1953b) shows that substrates of this enzyme occur only in minute amounts. Moreover, its inhibitor, ephedrine, is without effect on the ammonia formation of brain slices.

Adenylic and adenosine deaminases. It has been suggested by Embden and later by Parnas (see, for example, Parnas & Lewinski, 1935; Parnas & Lutwak-Mann, 1935) that the ammonia formation of working muscle is due to the activity of adenylic deaminase. This theory has been widely accepted, in spite of the fact that the agreement between the disappearance of adenosine phosphates and the formation of ammonia is not quantitative, since the sum of adenosine nucleotide amino-N and free NH_3 increases (Parnas & Lutwak-Mann, 1935). To explain this, the assumption had to be made that adenylic acid was taking part in a deamination-reamination cycle. Kalckar & Rittenberg (1947) found a rapid incorporation of ^{15}N into the amino group of muscle adenosine nucleotides when ammonium citrate labelled with ^{15}N was fed to rats and they interpreted this as confirmation of the existence of such a cycle. The possibility, however, has not been considered that the effect might be due to an exchange reaction between the adenylic amino group and free ammonium ions, catalysed by adenylic deaminase, in analogy to the exchange reactions catalysed by glutamine synthetase (Grossowicz, Wainfan, Borek & Waelsch, 1950; Stumpf, Loomis & Michelson, 1951; Elliott, 1953; Levintow & Meister, 1954). Wajzer & Nekhorocheff (1952) recently found an increase of the adenine fraction and a somewhat smaller disappearance of the hypoxanthine fraction during aerobic recovery of isolated frog muscle stimulated *in vitro*. Nekhorocheff & Wajzer (1952) and Nekhorocheff (1952) further claim that the reamination of inosinic acid

occurs at the expense of the $-\text{NH} \cdot \text{PO}_3\text{H}_2$ group of creatine phosphate, which is itself converted into urea. We have been unable to demonstrate any increased formation of ammonia after the addition of ITP or inosinic acid, with or without creatine phosphate, to brain slices or brain dispersions, washed, dialysed or untreated. In the absence of more definite evidence for the occurrence of the reamination reaction in brain it would be purely speculative to assume that the ammonia formation of brain slices involves a deamination-reamination cycle of adenosine nucleotides.

The concentration of preformed adenosine nucleotides in brain is of course far too small to account for the total of even the 'autolytic' fraction of the ammonia formation. According to the highest estimates their concentration is about $0.33 \mu\text{mole}/100 \text{ mg.}$ in the rat (Lindberg & Ernster, 1950) and the guinea pig (Kratzing & Narayanaswami, 1953), while with more specific enzymic methods a value of about $0.17 \mu\text{mole}/100 \text{ mg.}$ was obtained for rat brain (Albaum & Chinn, 1953). According to Tsukada & Takagaki (1954) the adenylic compounds did not decrease in guinea-pig brain slices during an incubation of 30 min.

We are thus led to the conclusion that there is no evidence which would allow one to ascribe the ammonia formation in brain slices to any of the five known deaminating systems occurring in brain.

In agreement with Astrup, Carlström & Stage (1951) we found no increase in the NPN of brain dispersions during an incubation of 5 hr. On the other hand, a significant increase was observed upon incubation of brain slices. This reaction therefore depends on the integrity of cellular structure in a way similar to the non-autolytic ammonia formation. Ansell & Richter (1954) have recently reported the existence of a very unstable neutral proteinase in brain which could not be extracted and which was only active for a short time after death. It is suggested, at present merely as a working hypothesis, that the ammonia formation of brain slices is connected with the activity of this enzyme.

The question as to whether the ammonia formation of brain slices is related to the ammonia formation induced by the stimulation of nervous tissues cannot be answered as yet. Geiger, Dobkin & Magnes (1953) have found that stimulation of cat brain cortex led to a marked increase of NPN. This would be in favour of a close connexion between the two processes. On the other hand, it may be pointed out that a similar and probably related process of ammonia formation occurs in other than nervous tissues, such as kidney, spleen, bone marrow, embryonic and neoplastic tissues (Warburg *et al.* 1924; Dickens & Greville, 1933; Lutwak-Mann, 1951). Most of these have a high glycolytic capacity (cf. Warburg *et al.* 1924).

The virtually complete suppression of the ammonia formation of brain slices in a glucose-containing medium still requires some comment. It is partly due to a lower degree of proteolysis, as shown by the NPN curves. The formation of acid-soluble phosphate and the fall of RNA-phosphate are similarly decreased in the presence of glucose. Glucose has therefore an inhibitory effect on catabolic reactions. The second factor is the operation of an ammonia-binding mechanism. Brain slices in glucose-saline form a considerable amount of amide N, no doubt at the expense of ammonium ions formed in a primary reaction. If the amide was formed by combination of ammonium ions with oxoglutaric acid, two moles of ammonium ions are fixed for each mole of amide N. Since an additional fraction of free ammonia may have disappeared in other reactions, e.g. in amination of oxoglutaric acid followed by transaminations to pyruvic or oxaloacetic acids, it is likely that, in relation to the amount of proteolysis, the primary ammonia formation is the same in the presence as in the absence of glucose.

SUMMARY

1. Slices of rat or guinea-pig brain in a glucose-free medium form ammonia at a fairly steady rate for at least 5 hr. During this period a final concentration of 3–4 μ moles/100 mg. tissue is reached.
2. The concentration of amide nitrogen does not change significantly during the aerobic or anaerobic incubation of brain slices.
3. The ammonia formation of brain slices is suppressed in glucose-saline. Instead, a synthesis of amide nitrogen takes place.
4. The ammonia formation of brain slices in glucose-free medium is inhibited under anaerobic conditions and by those agents that interfere with electron transport or oxidative phosphorylation.
5. The non-protein nitrogen and, to a lesser extent, the acid-soluble phosphorus of brain slices increase significantly during an incubation of 5 hr., particularly in a glucose-free medium.
6. A small, but probably significant, decrease occurs in the lipid phosphorus fraction and a relatively larger decrease in the ribonucleic acid phosphorus fraction of brain slices maintained in glucose-free medium. In glucose-saline the changes are smaller and not significant. The deoxyribonucleic acid phosphorus remains unchanged in the absence as well as in the presence of glucose.
7. Compared with brain slices, the initial ammonia concentration is higher and the final concentration lower when brain suspensions are incubated. In a hypotonic medium very little ammonia is formed upon incubation. In isotonic medium some ammonia is formed aerobically, but

not anaerobically. The addition of high-energy phosphate groups or of diphosphopyridine nucleotide is without effect on ammonia formation. No increase of non-protein nitrogen was found in brain suspensions.

8. Potential parent compounds were studied as to their effect on the ammonia formation of washed or dialysed brain suspensions and of brain mitochondria. These studies contributed nothing to the understanding of the mechanism of ammonia formation in slices.

9. It is concluded from the results that none of the five known deaminating brain enzymes is a major factor in the ammonia formation of brain slices and it is suggested that the ammonia arises in a reaction intimately linked with proteolysis.

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Ammonia Formation in Brain

2. BRAIN ADENYLIC DEAMINASE

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During our studies on the ammonia formation of brain slices (Weil-Malherbe & Green, 1955) the possibility was envisaged that adenylic deaminase might be the terminal enzyme, acting perhaps as a component of a reamination-deamination cycle. The study of this enzyme was originally undertaken in the hope that it might yield evidence bearing on this hypothesis.

Muntz (1953) recently described an unusual type of adenylic deaminase in aqueous extracts from acetone-dried dog brain. The enzyme was strongly activated by adenosine triphosphate, an effect that was greatest when the concentrations of adenosine triphosphate and adenylic acid were equal. The ammonia formation from adenylic acid alone was interpreted as being secondary to the conversion of adenylic acid into adenosine.

In the present paper observations on the purification of this enzyme and on the properties of the purified preparation are reported.

EXPERIMENTAL

Methods

Abbreviations. Adenosine triphosphate, adenosine diphosphate and 5'-adenylic acid are denoted by ATP, ADP and AMP. ITP, IDP and IMP stand for the corresponding

inosine phosphates. Ethylenediaminetetraacetate is denoted EDTA and trichloroacetic acid TCA.

Analytical. Enzyme activity was measured either by the estimation of ammonia or spectrophotometrically. Filtrates deproteinized with TCA at a final concentration of 5% (w/v) were used for the ammonia estimations, which were carried out by the microdiffusion method (Conway, 1950) and nesslerization; in some experiments the filtrates were analysed by direct nesslerization. The spectrophotometric method is based on the decrease of extinction at 265 m μ . and the increase at 240 m μ . when adenine nucleotides are deaminated to hypoxanthine nucleotides. In a solution with a nucleotide content corresponding to 1 μ g. adenine/ml. $\Delta E_{265 \text{ m}\mu} = -0.059$ and $\Delta E_{240 \text{ m}\mu} = +0.032$ (Kalckar, 1947). Since the enzyme preparations were turbid, the solutions were deproteinized with HClO₄ at a final concentration of 3% (w/v). The filtrates were diluted to contain $0.3\text{--}1 \times 10^{-4}$ M total nucleotides. A Unicam SP. 500 quartz spectrophotometer was used for the measurements.

Phosphate was estimated by a modification of the method of Martin & Doty (Weil-Malherbe & Green, 1951; Weil-Malherbe, 1953) and protein by the method of Lowry, Rosebrough, Farr & Randall (1951).

Standard conditions. For activity measurements the enzyme was incubated in a solution containing 0.0033 M AMP, 0.0033 M ATP, 0.02 M sodium succinate buffer (pH 6.2) and 0.01 M sodium EDTA (pH 6.8), in a total volume of 1.5 ml. or multiples thereof. The final pH of the