mucopolysaccharase activity which it has not so far been possible to remove.

3. The chondrosulphatase did not attack a number of other carbohydrate sulphate esters or esters which are known to be substrates for other types of sulphatases.

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Formation of Ammonia from Glutamine by Rat-brain Particles

By N. L. BLUMSOM

*Department of Biochemistry, University of Sheffield**

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Weil-Malherbe (1953) showed that in washed or dialysed rat-brain homogenates the rate of production of ammonia from glutamine was accelerated in the presence of inosine triphosphate (ITP) or of both inosinic acid and creatine phosphate. He suggested that the amide group of glutamine was transferred to ITP or another inosine phosphate to give the corresponding adenine compound, possibly at the expense of high-energy phosphate, followed by its deamination.

Reinvestigation of the problem with an enzyme preparation from rat brain apparently confirmed Weil-Malherbe's findings. However, further experiments failed to support his interpretation of the results.

It was noted that phosphate and sulphate stimulated production of ammonia under the conditions of the test (cf. Greenstein & Leuthardt,

1948). The apparent stimulation of production of ammonia from glutamine by inosine phosphates can be satisfactorily explained by the presence of phosphate and sulphate. These would be present in Weil-Malherbe's preparations. The effect of various ions on the reaction glutamine $\rightarrow$ glutamic acid + NH_3 was measured.

MATERIALS AND METHODS

Barium creatine phosphate was prepared by the method of Ennor & Stocken (1948), and the barium salt of inosinic acid (IMP) according to Ostern (1932) (see Lehninger, 1951). The barium salt of ITP was prepared according to Kleinzeller (1942). The preparation of the sodium salts from the barium salts was achieved by decomposition with sodium sulphate. Barium creatine phosphate was dissolved in water, and barium inosine mono- and tri-phosphates were dissolved in chilled N-HCl ; the barium was precipitated by the addition of a slight excess of $0.5\text{M-Na}_2\text{SO}_4$, the precipitate centrifuged off and the supernatant neutralized with NaOH (Eggleston

* Present address: Chemistry Building, King's College, College Walk, Newcastle upon Tyne, 1.

& Hems, 1952). Adenosine 5'-monophosphate (AMP), as the free acid, was a commercial sample (Roche Products Ltd.). Glutamine, prepared from mangolds (Hughes, 1950), was given by Dr D. E. Hughes. All solutions were brought to pH 7.4 before use.

³²P-Labelled IMP (Eggleston, 1954) was supplied by Mr L. V. Eggleston. The material was present on a square of chromatogram paper and gave about 30000 counts/min. The ³²P-labelled IMP was eluted from the paper for 10 min. by the reaction mixture in the vessel before the experiment, and the paper removed before addition of the enzyme.

Determination of ammonia. The microdiffusion method of Conway (1947) was used, the transfer time being 1 hr. at room temperature. When not in use the units were stored in 0.01 N-HCl. After diffusion of the NH₃ into the acid in the centre well, the contents were transferred to a glass-stoppered test tube, the well being washed out three times with 0.5–0.6 ml. of NH₃-free water, distilled in the apparatus described by Parnas & Heller (1924). The units were placed in a manipulator box (J. W. Towers Ltd., Widnes, Lancs.) containing an open dish of concentrated H₂SO₄, to remove traces of NH₃ from the atmosphere, during the transfer of the contents of the centre well to the test tubes. This precaution was necessary because atmospheric NH₃ caused inconsistencies. The volume was made up to 3 ml. after the addition of 0.03 ml. of 0.0003 M-MnSO₄. The NH₃ was estimated colorimetrically by the method of Russell (1945); 1 ml. of the alkaline phenoxide reagent and 1 ml. of the hypochlorite solution were added, and the colour was developed by standing the tubes in a boiling-water bath for 5 min. and then rapidly cooling them. The optical density at 625 mμ. was determined with a Unicam spectrophotometer against water.

Determination of phosphate compounds. Inorganic phosphate, ITP, IMP and AMP were separated and estimated by the method of Krebs & Hems (1953). The two solvents used were isopropyl ether–90% (w/v) formic acid (60:40, v/v) (Hanes & Isherwood, 1949) and isobutyric acid–NH₃ (isobutyric acid–N-NH₃ soln.–0.1 M disodium ethylenediaminetetraacetate; 100:60:1.6, by vol.).

Creatine phosphate (CP) was separated from inorganic phosphate by propan-1-ol–NH₃ [propan-1-ol–NH₃ soln. (sp.gr. 0.880)–0.1 M disodium ethylenediaminetetraacetate–water; 240:120:0.8:60, by vol.] (Oliver, personal communication). The solutions containing the CP were made alkaline after the precipitated protein had been removed by centrifuging and before 'spotting' on the paper.

Separation of glutamine and glutamic acid. This was achieved by the isobutyric acid–NH₃ solvent after 16–18 hr. on a descending chromatogram at room temperature. The compounds were located by spraying with 0.4% ninhydrin in *n*-butanol saturated with water, and heating.

Enzyme preparation. The brain of a freshly killed rat was removed and cooled in crushed ice. It was then weighed and homogenized with 5 ml. of cold water in a glass homogenizer of the Potter–Elvehjem type. To the homogenate was added sufficient water to make a concentration of 1 g. wet wt. of tissue to 10 ml. of water.

The mixture was spun in a Lusteroid centrifuge tube at 600 *g* for 3 min. in the high-speed head of an International refrigerated centrifuge. The supernatant was then spun at 8500 *g* for 15 min., the temperature being kept at 0°. The clear supernatant fluid was discarded and the particulate material resuspended in the original volume of water and

spun again at 8500 *g* for 15 min. The process was repeated and the resulting particles were suspended in water to serve as enzyme preparation. Dry weights of particle preparations were obtained at 105° after 4 hr. (5.2–5.8 mg./ml.).

Samples were incubated at 30°, in 20 ml. conical flasks fitted with rubber bungs and glass tubes for gassing. Each 4 ml. of reaction mixture contained 1.3 ml. of enzyme suspension and 0.4 ml. of 0.35 M-NaHCO₃ buffer, pH 7.4. The final concentration of IMP, AMP and CP was 0.004 M, of glutamine, 0.008 M. The gas space contained N₂+CO₂ (95:5).

Samples (1 ml.) were removed from the vessels at the desired times and deproteinized by adding to conical centrifuge tubes containing 0.1 ml. of 30% (w/v) trichloroacetic acid. The solutions were centrifuged to remove protein and the clear supernatant was stored at –14°.

The initial concentration of NH₃ in all reaction mixtures, 1–2 μg./ml., introduced by addition of the enzyme suspension and solutions, was subtracted from the values obtained after incubation.

RESULTS

Production of ammonia from glutamine in the presence of ITP or IMP and CP together

ITP or IMP and CP together stimulated formation of NH₃ on incubation with glutamine and the enzyme preparation (Table 1) (cf. Weil-Malherbe, 1953). No production of NH₃ occurred in the absence of glutamine, indicating that this substance is the primary source of the NH₃. The rates of hydrolysis of ITP, IMP and CP were measured and also the rates of production of NH₃ from these incubated mixtures. The results are shown in Figs. 1 and 2. Fig. 1 shows that the reaction rate decreased considerably when only a small proportion of the glutamine had been broken down.

Paper chromatography of samples of each reaction mixture showed that there was no perceptible formation of AMP or adenosine during the incubation. In those reaction mixtures where IMP was initially present, inorganic phosphate increased and a quantitatively corresponding amount of IMP

Table 1. *Production of ammonia from glutamine by washed brain particles*

Volume of reaction mixture was 4 ml., including 1.3 ml. of enzyme suspension. ITP, IMP and CP, 0.004 M; glutamine, 0.008 M; NaHCO₃, 0.035 M; pH 7.4. Temperature of incubation, 30°.

	NH ₃ produced in 60 min. (μg./ml.)
ITP + glutamine	28.2
IMP + CP + glutamine	20.1
IMP + glutamine	13.8
CP + glutamine	10.6
Glutamine	2.9
IMP + CP	0.0
IMP	0.0
CP	0.0
Nil	0.0

disappeared. In chromatograms from the same incubation mixture a spot appeared which absorbed the 257 $m\mu$. Hg line and ran a little behind the AMP area in the *isobutyric acid*- NH_3 solvent. This spot on elution gave an ultraviolet absorption curve with a peak at 248 $m\mu$., similar to that of hypoxanthine or inosine. The material ran in the same position as an inosine marker spot, but was different from both xanthine and hypoxanthine markers. Similar chromatographic evidence was obtained when propan-1-ol- NH_3 was the developing solvent. These findings were taken to indicate that the products of hydrolysis of IMP were inosine and inorganic phosphate. Chromatograms also showed that complete hydrolysis of the IMP by the enzyme preparation occurred after incubation for 4 hr. (see also Fig. 2).

Fig. 1. Rate of formation of ammonia from glutamine. Each vessel contained 4 ml. of reaction mixture, including 1.3 ml. of enzyme suspension, 0.008M glutamine and 0.035M- NaHCO_3 ; pH 7.4. Δ , No addition; $\circ$, 0.004M CP; $\blacktriangle$, 0.004M IMP; $\bullet$, 0.004M ITP.

Fig. 2. Rate of formation of orthophosphate. Each vessel contained 4 ml. of reaction mixture, including 1.3 ml. of enzyme suspension, 0.008M glutamine and 0.035M- NaHCO_3 ; pH 7.4. No addition resulted in no phosphate present. $\bullet$, 0.004M CP; $\circ$, 0.004M IMP; $\blacktriangle$, 0.004M ITP.

Further chromatographic examination with the *isobutyric acid*- NH_3 solvent showed that as the incubation proceeded glutamine decreased in amount and glutamic acid appeared.

Possibility of AMP being an intermediate in the reaction

Effect on adenylic deaminase activity of washing the brain particles. If AMP is an intermediate in the reaction, the activity of the adenylic deaminase present in the enzyme preparation must be sufficiently high to produce NH_3 from AMP at a rate equal to, or greater than, the rate of production from glutamine and ITP (or IMP and CP). To measure the rate of this formation of NH_3 , the crude homogenate, the once-washed particles and the twice-washed particles, were each incubated with AMP (final concn. 0.004M) under the test conditions. The results given in Table 2 show that the washing procedure reduced formation of NH_3 from AMP to negligible proportions. The rate and magnitude of production of NH_3 from a mixture of glutamine, IMP and CP, under the same conditions, were not affected.

Experiments with ^{32}P -labelled IMP

^{32}P Labelled IMP (0.004M), CP (0.004M), carrier AMP (0.004M) and glutamine (0.008M) were incubated with the suspension for 60 min. Samples of the reaction mixture at zero time and at 60 min. were run on chromatograms and the AMP areas cut out, wet-ashed and counted as described by Eggleston & Hems (1952). There was no increase with time of the radioactivity associated with the AMP area. This showed that IMP had not been aminated, although 16 μg . of NH_3 /ml. had been formed.

If the hydrolysis of AMP was inhibited, any AMP formed from IMP and glutamine would accumulate and could be detected. Heppel & Hilmo (1951) showed that low concentrations of NaF inhibited 5'-nucleotidase. Paper chromatography indicated that NaF at a final concentration

Table 2. *Effect of washing rat-brain particles on adenylic deaminase activity and production of ammonia from glutamine*

Volume of reaction mixture was 3 ml., including 1.3 ml. of enzyme suspension; AMP, IMP and CP, 0.004M; NaHCO_3 , 0.035M; pH 7.4. Temperature of incubation, 30°.

Enzyme suspension	NH_3 ($\mu\text{g./ml.}$) produced in 60 min. from	
	AMP	Glutamine + IMP + CP
Whole homogenate	12.1	20.6
Once-washed particles	3.0	19.8
Twice-washed particles	0.6	19.5

of 0.02M inhibited the hydrolysis of AMP and IMP completely, under the test conditions. No increase in inorganic phosphate was detected even after incubation for 4 hr. The previous experiment was repeated with NaF added to a final concentration of 0.02M. After incubation for 4 hr. there was no increase in radioactivity associated with the AMP area.

Effect of L-glutamic acid on production of NH_3 from glutamine

Krebs (1935) found that production of NH_3 from glutamine by brain glutaminase was inhibited equally by addition of either L- or D-glutamic acid. This observation might explain the decline in the rate of production of NH_3 , with time, described above. Table 3 shows that formation of NH_3 from glutamine in the presence of IMP, CP and the brain preparation is inhibited 97% by L-glutamic acid.

Effect of phosphate and sulphate on production of NH_3 from glutamine

Greenstein & Leuthardt (1948) found that certain ions, especially phosphate and sulphate, activated the glutaminase of rat-brain homogenates.

The preparation of solutions of CP, IMP and ITP involved decomposition of the barium salts with an excess of sodium sulphate. Thus solutions of these substances always contain sulphate. During incubation IMP undergoes hydrolysis and yields phosphate. It was considered that the increased production of NH_3 during incubation of glutamine with IMP and CP might therefore be due to glutaminase activation by sulphate and phosphate.

To investigate this possibility, phosphate equivalent in amount to the IMP usually added was incubated with glutamine and the enzyme. A second vessel also contained sulphate (0.002M final concn.). This amount was added to allow for sulphate that had been added for complete precipitation of barium from IMP and CP, and was about 17% in excess. Table 4 shows the results of production of NH_3 in a typical experiment.

The figures show that both phosphate and sulphate stimulate production of NH_3 at low concentration and could explain the effect of addition of IMP and CP.

Effect of various ions on production of NH_3 from glutamine

Phosphate. A range of phosphate concentrations, 0.00125–0.08M, was tested; the results in Fig. 3 show that formation of NH_3 increased with phosphate concentration and had not reached a maximum at 0.08M.

The effect of glutamic acid on this stimulation was also tested. Two levels of phosphate concentration were used and incubated with glutamine and

enzyme under the test conditions. At the same time a similar experiment was run where glutamic acid was also added at half of the concentration of the glutamine present. Fig. 4 shows that doubling the concentration of phosphate increased the initial rate of formation of NH_3 , over the blank value, by a factor of 2.7. In the presence of the glutamic acid the rate of formation of NH_3 was much reduced. In the absence of added inorganic phosphate glutamic acid inhibited production of NH_3 completely.

Other ions. A variety of other ions was tested at a concentration of 0.013M. The results are given in

Table 3. *Effect of glutamic acid on production of ammonia*

Volume of reaction mixture was 4 ml., including 1.3 ml. of enzyme suspension. IMP and CP, 0.004M; glutamine, 0.008M; L-glutamic acid, 0.004M; NaHCO_3 , 0.035M; pH 7.4. Temperature of incubation, 30°.

	NH_3 produced in 60 min. ($\mu\text{g./ml.}$)
IMP + CP + glutamine	18.9
IMP + CP + glutamine + glutamic acid	0.6

Table 4. *Effect of phosphate and sulphate on production of ammonia from glutamine*

Volume of reaction mixture was 4 ml., including 1.3 ml. of enzyme suspension. IMP and CP, 0.004M; Na_2HPO_4 , 0.004M; Na_2SO_4 , 0.002M; NaHCO_3 , 0.035M; pH 7.4. Temperature of incubation, 30°.

	NH_3 produced in 60 min. ($\mu\text{g./ml.}$)
IMP + CP + glutamine	15.9
CP + glutamine	10.6
$\text{PO}_4^{3-} + \text{SO}_4^{2-} + \text{glutamine}$	14.3
$\text{PO}_4^{3-} + \text{glutamine}$	9.4
Glutamine	2.9

Fig. 3. Effect of increasing phosphate concentration on production of ammonia from glutamine. Each vessel contained 4 ml. of reaction mixture, including 1.3 ml. of enzyme suspension, 0.008M glutamine, 0.035M NaHCO_3 ; pH 7.4. Phosphate concentrations varied as shown. Time of incubation, 60 min.

Table 5. Pyrophosphate caused an increase in production of NH_3 nearly double that caused by the same concentration of phosphate. The pyrophosphate was analysed by paper chromatography with formic acid-isopropyl ether as the developing solvent; no hydrolysis to inorganic phosphate occurred.

Soluble enzyme

Several attempts were made to prepare a soluble enzyme preparation. The suspension of rat-brain particles in either water or bicarbonate buffer (0.035M) was shaken with ballotini beads in a

Fig. 4. Effect of L-glutamic acid on the rate of production of ammonia from glutamine in the presence of different concentrations of orthophosphate. Each vessel contained 4 ml. of reaction mixture, including 1.3 ml. of enzyme suspension, 0.008M glutamine and 0.035M- NaHCO_3 ; pH 7.4. ■, No additions; ○, 5 μmoles of phosphate/ml.; △, 10 μmoles of phosphate/ml.; 4 μmoles of L-glutamic acid resulted in no ammonia production; ▲, 4 μmoles of glutamic acid + 5 μmoles of phosphate/ml.; ●, 4 μmoles of glutamic acid + 10 μmoles of phosphate/ml.

Table 5. *Effect of various salts on glutaminase of rat brain*

Enzyme suspension, 1.3 ml. Substrate, glutamine. Concentration of salt, 0.013M; HCO_3^- , 0.035M; pH 7.4. Temperature of incubation, 30°.

Salt added	NH_3 formation ($\mu\text{g.}/\text{ml.}/120 \text{ min.}$)
Nil	3.7
Na_2HPO_4	40.6
Na_2SO_4	23.6
NaBO_3	12.8
$\text{Na}_2\text{S}_2\text{O}_3$	16.3
KIO_3	5.5
$\text{Na}_4\text{P}_2\text{O}_7$	77.0
NaF	5.1
Na_2HASO_4	14.7
$\text{K}_4\text{Fe}(\text{CN})_6$	22.2
$\text{K}_3\text{Fe}(\text{CN})_6$	1.1
Sodium ethylenediaminetetraacetate	8.3
MgCl_2	8.6

Mickle vibrator for 15 min. in the cold room. The clear supernatant obtained on centrifuging was tested for enzyme activity under the conditions described above but was found inactive. The sediment was also inactive, as was the recombined supernatant and sediment.

DISCUSSION

The incubation of IMP and CP or ITP (Table 1) with glutamine in the presence of washed rat-brain particles gives a greater production of ammonia than is found with glutamine alone. It has also been shown that the added glutamine is the primary source of the ammonia.

Washing the rat-brain particles resulted in a very marked decrease in ability to deaminate AMP (Table 2), whereas the rate and magnitude of production of ammonia from glutamine, CP and IMP under the same conditions was not affected. Further, when these twice-washed particles were incubated with glutamine, CP, ^{32}P -labelled IMP and carrier AMP there was no incorporation of radioactivity into the AMP, but production of ammonia was unaffected. In fact, no evidence for the amination of IMP to form AMP was found under conditions where IMP and CP stimulated the formation of ammonia from glutamine. This was taken to imply that the mechanism suggested by Weil-Malherbe (1953) for this stimulation, involving the intermediate amination of IMP, was not operative. Another explanation was sought.

Greenstein & Leuthardt (1948) had shown that rat-brain glutaminase was activated by very low concentrations of ions, especially phosphate and sulphate. Production of ammonia from glutamine under the test conditions could be explained satisfactorily in this manner.

The solution of ITP contained a little inorganic phosphate initially as can be seen from Fig. 2. It was found that both IMP and ITP were hydrolysed by the enzyme preparation to yield inorganic phosphate. The rate of hydrolysis of CP was very much slower.

The preparation of the solutions of the sodium salts of these compounds is achieved by precipitating the barium from solution with a slight excess of sodium sulphate, as a result of which these solutions always contain some sulphate.

The effect of inorganic phosphate and sulphate at low concentrations (0.004M) was tested on the system deaminating glutamine; both were found to stimulate production of ammonia. The combined stimulation of these ions at this concentration was approximately equal to that of adding the same concentration of IMP and CP.

The effect of addition of glutamic acid to the enzyme system deaminating glutamine in the

presence of IMP and CP resulted in about 97 % inhibition of production of ammonia. This result would be expected if the enzyme responsible were the brain glutaminase described by Krebs (1935). With inorganic phosphate (5 or 10 μ moles/ml.) replacing the IMP and CP in the system, addition of glutamic acid (4 μ moles/ml.) was accompanied by a very marked decrease in production of ammonia.

Greenstein & Leuthardt (1948) found that bicarbonate buffer did not activate glutaminase. Further evidence for this has been found in the low blank values. They measured the stimulatory effect of various ions on glutaminase, and some organic derivatives of phosphate and arsenate. The present study has included more multivalent ions, since Greenstein & Leuthardt (1948) had shown that univalent ions were poor stimulators of the reaction. It was concluded that, in general, as the ionic charge increased so did the effectiveness in stimulating production of ammonia (Table 5).

The effect obtained by Weil-Malherbe (1953) can be readily explained on the basis of an anion activation of brain glutaminase by phosphate formed by hydrolysis during the incubation, or by phosphate and sulphate added initially in these solutions.

SUMMARY

1. The formation of ammonia from glutamine by rat-brain particles in the presence of inosine triphosphate, or inosinic acid and creatine phosphate, is explained by the presence of low concentrations of phosphate and sulphate which activate the glutaminase present.

2. The reaction is 97 % inhibited by the addition of L-glutamic acid.

3. The effect of various other ions on the reaction was studied. The results indicated that, as the ionic charge increases, so does the efficacy in stimulating production of ammonia.

4. The synthesis of adenosine 5'-monophosphate from inosinic acid does not occur in this system under these conditions.

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The Purification and Properties of Arginine Phosphokinase

By J. F. MORRISON, D. E. GRIFFITHS* AND A. H. ENNOR

Department of Biochemistry, John Curtin School of Medical Research, The Australian National University, Canberra, A.C.T., Australia

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Phosphocreatine and phosphoarginine have been known for many years, but in spite of their importance in the process of muscular contraction little attention has been paid to the enzymes involved in their metabolism. The first detailed studies of creatine phosphokinase were made by Noda, Kuby & Lardy (1954), Kuby, Noda & Lardy (1954) and Ennor & Rosenberg (1954). The enzyme

arginine phosphokinase was first found to be present in crude extracts of crab muscle by Lohmann (1935), who showed that it was responsible for catalysing the equilibrium as expressed by the reaction phosphoarginine + adenosine diphosphate $\rightleftharpoons$ arginine + adenosine triphosphate. The presence of the enzyme in extracts of octopus muscle and the jaw muscles of the echinoid *Sphaerechinus granularis* was demonstrated by Lohmann (1936) and by Baldwin & Needham (1937) respectively. Soreni, Dvornikova

* Australian National University Research Scholar.