EVIDENCE FOR A "NEUTRAL PROTEINASE" IN BRAIN TISSUE

by

G. B. ANSELL AND D. RICHTER

Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff (Wales)

In the course of work on the amino acids in brain it was observed that the amino-acid content of fresh brain tissue increases considerably on brief incubation at pH 7.4. Intracellular proteinases generally function optimally at pH 3–5, but there have been occasional references in the literature to proteinases active at neutral pH (HEDIN[1]; DERNBY[2]; ADAMS AND SMITH[3]). BELOFF AND PETERS[4] were able to extract an enzyme that broke down casein and other proteins at pH 7.0. from rat skin, kidney, liver and muscle; but they did not obtain it from brain tissue and a "neutral proteinase" has not hitherto been reported in nervous tissues. KIES AND SCHWIMMER[5] found no proteolytic activity in calf brain at pH 7.5 and ASTRUP, CARLSTRÖM AND STAGE[6] have recently found no increase in the non-protein nitrogen of brain tissue autolysed for 48 hr.

In this paper evidence is presented of a system in brain tissue that shows considerable proteolytic activity at pH 7.4. The system is unstable and can therefore be detected only in fresh brain tissue examined a short time after death.

METHODS

Incubation of tissue

Rats were killed by decapitation and the brains immediately removed and chilled on ice. The tissue, minced sufficiently fine for pipetting, was diluted with cold KREBS phosphate-Ringer solution[7] to make a suspension containing 250 mg brain tissue per ml. The suspension (1 ml) was transferred to Warburg vessels containing 2 ml phosphate-Ringer solution. The vessels were gassed with nitrogen after introducing 0.1 g yellow phosphorus into the centre cups, and incubated at 37°. The incubation started 25–30 min after the death of the animal.

Amino-nitrogen determination

Trichloroacetic acid (3 ml 20% solution) was added to each vessel. The supernatant solution obtained on centrifuging was heated at 70° for 75 min to convert glutamine to pyrollidone carboxylic acid and ammonia. The solution was extracted twice with an equal volume of ether to remove excess trichloroacetic acid. Control experiments showed that this extraction did not remove amino-nitrogen. This was determined by the method of MOORE AND STEIN[8]. To 0.125 ml of the solution was added 0.375 ml 0.2 *M* citrate buffer (pH 5.0) and 1 ml of the ninhydrin-stannous chloride reagent. After heating for 20 min at 100° in a glass stoppered tube the contents were diluted to 10 ml with 50% v/v *n*-propanol and the extinction coefficient at 570 mμ determined with a photoelectric absorptiometer. Glutamic acid was used as a standard for comparison. The results were corrected for the colour due to the ammonia content, which was determined separately by the method of CONWAY[9]. Estimations were carried out in a similar manner on acid-hydrolysates prepared by heating 1 ml brain extract with 1 ml 11 *N*-hydrochloric acid for 24 hr at 100° in a sealed tube. The hydrolysate was evaporated to dryness in a vacuum desiccator containing phosphorus pentoxide and sodium hydroxide, and then diluted to 1 ml.

References p. 97.

Paper chromatography

Two-dimensional paper chromatograms were prepared on Whatman No. 4 paper, using water-saturated phenol and collidine-lutidine as solvents. The solutions were desalted by the method of CONSDEN *et al.*[10] and a volume equivalent to 20 mg brain tissue was generally used. The amino-compounds were revealed by spraying with 0.1% ninhydrin in water-saturated *n*-butanol or 0.2% ninhydrin in 95% ethanol. The copper treatment of CRUMPLER AND DENT[11] was used to differentiate other amino-compounds from α-amino acids which were identified with markers in the usual way.

RESULTS

Proteolytic activity at pH 7.4

The protein breakdown was followed by measuring the rate of liberation of free amino acids. When rat brain slices or a suspension of fresh rat brain tissue was incubated anaerobically at 37° and pH 7.4 there was a rapid liberation of amino-nitrogen which ceased within 1–1½ hours after death (Fig. 1). The free amino-nitrogen showed no

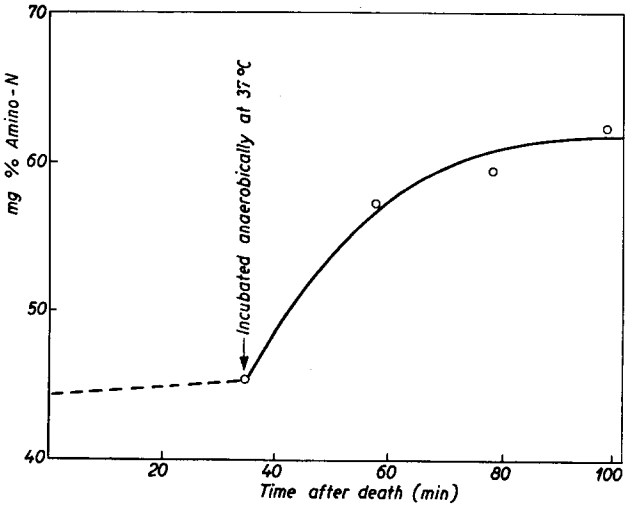

Fig. 1. The liberation of amino-nitrogen from minced rat brain incubated at 37°.

appreciable change during the preparation or the suspension if the tissue was kept at 0°. The amount of amino-nitrogen released on incubating for 1 hour at 37° ranged from 6.8–25.3 mg% with a mean of 17.1 mg% in 10 different rat brain preparations. The initial rate of amino-nitrogen liberation was approximately 35 mg% per hour, which corresponds to 1.8 μg N/mg dry weight/hour. The system is also present in pigeon and rabbit brain. A comparison of the relative activities in the grey and white matter of the rabbit brain showed that the activity in white matter is 75% greater than that in the grey.

Amino acids liberated

Paper chromatograms of extracts prepared from rat brain suspensions confirmed that there was a generalised increase in the free α-amino acids on incubation at 37° at pH 7.4. Increases were observed in the concentrations of aspartic acid, glutamic acid, glycine, serine, alanine, lysine, arginine, valine and a mixture giving a spot in the position of the leucine isomers. A further chromatographic separation by the method

References p. 97.

of WORK[12] showed that both leucine and isoleucine were released. Approximate figures for the amount of the individual amino acids liberated were obtained by the method of ROBERTS AND FRANKEL[13]. It was found in three experiments that the increase in 1 hour in aspartic acid ranged from 10–30%, glutamic acid 0–10%, glycine 100–200%, serine 60–100% and alanine 50–100%.

Peptides in brain tissue

It appeared possible that the amino acids released on incubation might be derived from peptides or polypeptides in the tissue through the action of the peptidases which are known to be present. In order to test this, the peptide-bond nitrogen was estimated in brain suspensions before and after incubation. The peptides were estimated by determining the increase in amino-nitrogen after hydrolysis of the extracts with 5.5 *N*-hydrochloric acid for 24 hours at 100°. A correction was made for the increase due to the hydrolysis of pyrollidone carboxylic acid to glutamic acid. It was found by paper chromatography that there was no appreciable increase in the glutamine fraction on incubation: a correction of 7 mg % based on the observed glutamine α-amino-nitrogen content was therefore made for this factor. Values were obtained in this way for the peptide-bond nitrogen in (a) rat brain obtained after rapid freezing in liquid oxygen, (b) suspensions of rat brain kept at 0° and examined 25–30 min after death, and (c) rat brain suspensions prepared similarly but incubated for 60 minutes anaerobically at 37° and pH 7.4. It can be seen from Table I that the peptide-bond nitrogen remained constant during the preparation of the brain suspension and during the incubation, although the free amino-nitrogen increased. The liberated amino-nitrogen could not therefore be derived from peptides or polypeptides in the brain tissue except in so far as they were broken down and replaced.

TABLE I

THE EFFECT OF INCUBATION AT 37° AND pH 7.4 ON THE PEPTIDE-BOND NITROGEN CONTENT OF RAT BRAIN SUSPENSIONS

No of experiments	*Treatment of brain tissue*	*Time of incubation at 37° (min)*	*Mean amino-N liberated (mg %)*	*Mean peptide-bond-N content (mg %)*
2	Frozen immediately in liquid oxygen	0	0	19
5	Tissue at 0° minced and suspended in phosphate-Ringer solution	0	2	23
4	Tissue at 0° minced and suspended in phosphate-Ringer solution	60	22	22

Stability of proteolytic system

The rapid cessation of proteolytic activity in brain tissue at pH 7.4 after 1–1½ hours might be attributed to the accumulation of end-products. In order to test this, the amino-nitrogen liberated from a rat brain suspension (375 mg tissue in 5 ml phosphate-Ringer solution) after incubation for three hours at 37° was compared with the amount liberated from a similar suspension incubated in a cellophane dialysis tube immersed in 50 ml phosphate-Ringer solution. It was found that the removal of the

References p. 97.

end-products in this way did not affect the amount of amino-nitrogen liberated or prolong the activity of the system.

Conditions of optimal activity

The effect of pH on the activity of the system was determined with aqueous rat brain suspensions adjusted to the correct pH by the addition of SØRENSEN[14] phosphate buffers immediately prior to anaerobic incubation at 37°. The brain suspension (250 mg in 1 ml) was put in the side-arm of a THUNBERG tube containing nitrogen and 2.5 ml 0.2 *M* phosphate buffer. The contents were mixed 30 minutes after death of the animal and incubated for 60 minutes. In control experiments the pH values were checked with a glass electrode after mixing. The quantities of amino-nitrogen liberated at pH 6.64, 6.98, 7.38 and 8.04 respectively were 11, 5, 17 and 13 mg % in 60 minutes. The maximum liberation of amino-nitrogen thus occurred near the physiological pH of 7.4. The liberation of amino-nitrogen was not affected by the addition of a boiled brain suspension. The activity of the system did not therefore depend on the presence of thermo-stable activators or denatured protein. The brain suspensions were estimated to contain about 0.1% of blood, a plasma component of which is known to inhibit the "neutral proteinase" of skin[15]. Experiments in which up to 1.7% of whole blood, blood plasma and erythrocytes were added to the incubated brain suspension showed that these additions did not greatly affect the activity of the system.

The activity of the rat brain system was not appreciably affected by (a) 0.01 *M* cysteine, (b) 0.01 *M* cyanide, (c) $0.03 \cdot 10^{-3}$ *M* ferrous sulphate and 0.0014 *M* ascorbic acid[16], or (d) $0.18 \cdot 10^{-3}$ *M* $\alpha\alpha'$-dipyridyl and 0.0014 *M* ascorbic acid[16], which are known to activate or inhibit other proteolytic enzymes. It was 97% inhibited by (e) 0.01 *M* iodoacetate and 95% by (f) 0.005 *M* copper sulphate. Control experiments showed that this concentration of copper did not interfere with the estimation of added amino acids.

The system showed no activity in extracts prepared by extracting fresh brain tissue or "acetone powders" of rat brain tissue with 50% v/v glycerol-water mixture. The activity of the extracts at pH 7.5 was tested by the method of ANSON[17] with urea-denatured hemoglobin as substrate. Attempts to extract the enzyme from a brain homogenate with Ringer solution or with 0.1 *M* phosphate buffer pH 7.4 and *n*-butanol, as described by MORTON[18], were also unsuccessful.

DISCUSSION

It has been shown that fresh brain tissue contains a system that liberates amino acids on brief incubation at pH 7.4. The system is unstable and active for only about 1–1½ hours after death. The amino acids are not derived from a simple peptide or polypeptide, for it was shown that at least nine different amino acids are liberated under these conditions, and the peptide-bond nitrogen in the tissue did not decrease. It is concluded that the amino acids are liberated through the action of an intracellular proteinase active at pH 7.4. The system is not activated by cysteine or cyanide; it is strongly inhibited by iodoacetate and by copper ions.

The amount of amino-nitrogen liberated by the system in 1 hour was of the order of 17 mg % in rat brain tissue. If it is assumed that this was derived from a protein containing 16% total nitrogen and each protein molecule was completely broken down, this would represent a breakdown of an amount of the order of 100 mg % protein in

References p. 97.

1 hour. The activity of the system decreased rapidly during the course of the incubation. The initial rate of amino-nitrogen liberation was approximately 35 mg % per hour, which corresponds to a liberation of 1.8 μg amino-N/mg dry weight/hour. This rate is much higher than has been reported previously for proteolysis under any conditions in brain tissue: it may be compared with the values 0.015 μg N/mg/hour for sheep brain cathepsin during autolysis at pH 1.7 and 0.042 μg N/mg/hour for calf brain cathepsin at pH 3.6, which may be calculated from the figures of GIBSON *et al.*[19] and KIES AND SCHWIMMER[5].

The rapid falling off in the activity of the system during incubation at 37° was not due to the accumulation of end-products, since their removal by dialysis did not prolong the activity. The apparent instability of the system could be due to a number of factors, including the destruction of activators. It is significant that ABDERHALDEN AND CESAR[20] found that the brain polypeptidase was also unstable under similar conditions.

The system differs from the brain cathepsin in pH optimum, stability, extractibility, behaviour towards inhibitors and distribution in the grey and white matter of the brain. It differs from the skin proteinase of BELOFF AND PETERS[4] in stability and in that it is not inhibited by blood plasma. It differs also from cathepsin III in that it is not activated by ferrous ions, nor is it inhibited by $\alpha\alpha'$-dipyridyl[16]. Evidence is accumulating that animal tissues contain intracellular proteinases, active at physiological pH values and differing considerably in their properties from the more widely investigated cathepsins. It is likely that they are concerned in the continual breakdown and resynthesis of the tissue proteins *in vivo*. The "neutral proteinase" of brain tissue probably falls into this category.

ACKNOWLEDGEMENTS

The authors thank the Medical Research Council for a grant to one of us (G.B.A.); they thank Mr. R. B. WILLIAMS for carrying out the initial experiments which led to this investigation and Mr. D. W. DAVIES for his technical assistance.

SUMMARY

1. Fresh brain tissue contains an active proteinase that liberates amino acids on incubation at pH 7.4. It is unstable and becomes inactive within about 1–1½ hours after death.

2. It was shown by paper chromatography that at least nine different amino acids are liberated by the system. They are not derived from peptides or polypeptides present in the brain tissue.

3. The proteinase is not activated by 0.01 *M* cysteine or 0.01 *M* KCN: it is almost completely inhibited by 0.01 *M* iodoacetate and by 0.005 *M* copper sulphate.

4. The system is 75% more active in the white matter than in the grey matter of the rabbit brain. The initial activity in the rat brain corresponds to a liberation of 1.8 μg amino-N/mg dry weight/hour.

RÉSUMÉ

1. Le tissu cérébral frais renferme une protéinase active qui libère des acides aminés après incubation à pH 7.4. Cette protéinase est instable et s'inactive environ 1 heure à 1 heure ½ après la mort.

2. La chromatographie sur papier révèle au moins neuf acides aminés différents libérés par ce système. Ils ne proviennent pas de peptides ou de polypeptides présents dans le tissu cérébral.

3. La protéinase n'est pas inactivée par la cystéine 0.01 *M* ou le KCN 0.01 *M*; elle est presque complètement inhibée par l'iodoacétate 0.01 *M* et par le sulfate de cuivre 0.005 *M*.

4. Chez le lapin, l'activité dans la matière blanche est supérieure de 75% à l'activité dans la matière grise. L'activité initiale chez le rat correspond à la libération de 1.8 μg de N aminé/mg poids sec/heure.

References p. 97.

ZUSAMMENFASSUNG

1. Frische Gehirngewebe enthalten eine aktive Proteinase, die bei Bebrütung bei pH 7.4 Aminosäuren in Freiheit setzt. Sie ist instabil und wird 1–1½ Stunden nach dem Tode inaktiv.

2. Mit Hilfe der Papierchromatographie wurde gezeigt, dass mindestens 9 verschiedene Aminosäuren von den System in Freiheit gesetzt werden. Sie stammen nicht von den in den Gehirngeweben anwesenden Peptiden und Polypeptiden.

3. Die Proteinase wird nicht von 0.01 *M* Cystein oder 0.01 *M* KCN aktiviert. Sie wird beinahe vollständig von 0.01 *M* Jodacetat und von 0.005 *M* Kupfersulfat gehemmt.

4. Das System ist um 75% aktiver in der weissen als in der grauen Gehirnsubstanz beim Kaninchen. Die anfängliche Aktivität im Rattengehirn entspricht einer Freisetzung von 1.8 μg Aminostickstoff/mg Trockengewicht/Stunde.

REFERENCES

1 S. G. HEDIN, *J. Biol. Chem.*, 54 (1922) 177.
2 K. G. DERNBY, *J. Biol. Chem.*, 35 (1918) 179.
3 E. ADAMS AND E. L. SMITH, *J. Biol. Chem.*, 191 (1951) 651.
4 A. BELOFF AND R. A. PETERS, *J. Physiol.*, 103 (1945) 461.
5 M. W. KIES AND S. SCHWIMMER, *J. Biol. Chem.*, 145 (1942) 685.
6 T. ASTRUP, G. CARLSTRÖM AND A. STAGE, *Acta physiol. scand.*, 24 (1951) 202.
7 P. P. COHEN in UMBREIT *et al.*, *Manometric techniques and related methods for the study of tissue metabolism*. Burgess Publishing Co., Minneapolis, 1st. ed. (1945) 191.
8 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 367.
9 E. J. CONWAY, *Microdiffusion analysis and volumetric error*. Crosby, Lockwood and Son Ltd., London (1939).
10 R. CONSDEN, A. H. GORDON AND A. J. P. MARTIN, *Biochem. J.*, 41 (1947) 590.
11 H. R. CRUMPLER AND C. E. DENT, *Nature*, 164 (1949) 441.
12 E. WORK, *Biochim. Biophys. Acta*, 3 (1949) 400.
13 E. ROBERTS AND S. FRANKEL, *J. Biol. Chem.*, 187 (1950) 55.
14 S. P. L. SØRENSEN, cited by A. M. CLARK, *The determination of hydrogen ions*. Williams and Wilkins Co., Baltimore, 2nd. ed. (1923).
15 A. BELOFF, *Biochem. J.*, 40 (1946) 108.
16 I. KRIMSKY AND E. RACKER, *J. Biol. Chem.*, 179 (1949) 903.
17 M. L. ANSON, *J. gen. Physiol.*, 22 (1938/9) 79.
18 R. K. MORTON, *Nature*, 166 (1950) 1092.
19 C. A. GIBSON, F. UMBREIT AND H. C. BRADLEY, *J. Biol. Chem.*, 47 (1921) 333.
20 E. ABDERHALDEN AND G. CESAR, *Fermentforschung*, 16 (1940) 255.

Received July 4th, 1953