THE PROTEOLYTIC ACTIVITY OF BRAIN TISSUE

by

G. B. ANSELL AND D. RICHTER

Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff (Wales)

KIES AND SCHWIMMER[1] reported the presence of a powerful cathepsin in calf brain: the activity per mg protein was about twelve times greater than in muscle. They found it acted optimally at pH 3.5 and it showed no activity at pH 7.5. It is hard to conceive the *in vivo* function of an enzyme that is optimally active so far from the normal physiological pH range; but FRUTON[2] and his collaborators have recently shown that cathepsins can also act at the neutral pH as catalysts of transamidation reactions. This suggests that they may be concerned with protein synthesis rather than with proteolysis under normal conditions *in vivo*.

The object of this investigation was to determine the activities of the cathepsin in human and other brain tissue and find out more about the properties of the enzyme. If the brain cathepsin is concerned with protein synthesis it would be expected to be located mainly in the nerve cell bodies or in the cell nuclei, where protein synthesis is believed to be particularly active[3].

METHODS

Materials

The human brains were taken at autopsy about 24 hr after death from cardiac failure. The meninges were removed and the cortex carefully dissected from the underlying white tissue. Purified brain cathepsin was prepared from an "acetone powder" of human brain cortex by extracting it with 0.2 *M* acetic acid, precipitating with 80% ammonium sulphate and dialysing a suspension of the precipitate as described by KIES AND SCHWIMMER[1]. It formed a clear solution which could be kept for several weeks at 4°.

Isolated nuclei were prepared from rabbit and human brain by the method of DOUNCE[4] under the conditions described by RICHTER AND HULLIN[5]. It has been reported that a part of the nuclear protein is lost when nuclei previously extracted with lipid solvents are suspended in citrate solution[6]; but there is no evidence of any serious loss from brain cell nuclei separated under the conditions described.

The peptone solution used in testing for polypeptidase activity was prepared from a commercial sample of "bacteriological peptone" (Baird and Tatlock). This was purified by saturating the solution with ammonium sulphate, discarding the precipitate and dialysing the supernatant solution. It gave a pink colour in the biuret test and a precipitate with tungstic and tannic acids. Nine different amino acids were identified chromatographically in the acid hydrolysate.

Measurement of cathepsin activity

The cathepsin activity was measured by determining the amount of tyrosine and tryptophan liberated in 20 minutes by the action of the enzyme on a solution of denatured hemoglobin, as described by ANSON[7]. Hemoglobin freshly prepared from ox blood, was denatured by incubating 4 ml quantities of a 7.5% solution for 20 min at 37° with 1 ml of a solution containing 0.02 *M* ammonium sulphate and 1.35 *M* acetic acid; the mixture gave a pH of 3.8. The tissue suspension or enzyme solution (1 ml) was added and the reaction stopped after 20 min by adding 10 ml 5% tri-

References p. 91.

chloroacetic acid. After standing 10 min the solution was filtered and the tyrosine and other colour-forming substances were estimated in a 5 ml quantity by adding 10 ml 0.5 *N* sodium hydroxide followed by 3 ml of a 33% dilution of Folin and Ciocalteu reagent. The colour was measured with a photoelectric colorimeter using an Ilford tri-colour red filter No. 204, and a standard curve relating Anson cathepsin units to extinction was constructed. An unincubated mixture of the same components served as a blank. It was confirmed with standard tyrosine solutions that the colour intensity obeyed the BEER-LAMBERT law under the conditions used. The ANSON unit is defined as the amount of cathepsin which liberates from denatured hemoglobin under standard conditions an amount of split products giving the same colour intensity with Folin reagent as 1 milliequivalent of tyrosine[7].

RESULTS

It was readily shown that a cathepsin is present in the brain tissue of other animals besides the calf. The activity in human brain was about five times greater on a dry weight basis in the grey matter of the cerebral cortex than in the white matter.

TABLE I

ACTIVITY OF BRAIN CATHEPSIN IN DIFFERENT ANIMALS

Animal	*Tissue*	*Cathepsin units/g wet weight* $\times 10^{-4}$	*Cathepsin units/g dry weight* $\times 10^{-4}$
Rat	whole brain	6.4	32
Cow	cortex	10	52
Rabbit	cortex	6	31
Man	white matter	3.6	12
Man	cortex	11	72

Stability

Determinations of the cathepsin activity of rat and rabbit brain tissue showed that there was no decline in activity on keeping at 4° for periods up to 27 hours: the activities were identical with those found in brain suspensions incubated within 10 minutes after death. The brain suspensions were prepared by homogenising 6 g tissue with 30 ml water and 0.1 ml capryl alcohol in a Waring blender. They were kept at 4° in the presence of toluene. Measurements of cathepsin activity showed no significant loss of activity in periods up to 7 days. There was no evidence of any rapid post mortem inactivation of the enzyme and it therefore appeared suitable for study in autopsy material.

The purified cathepsin prepared from a brain "acetone powder" by precipitating the extract with ammonium sulphate followed by dialysis, showed some falling off in activity when the solution was incubated at 37°. The loss of activity, which amounted to 25–30% in two hours at 37°, was not prevented by the addition of 0.09 *M* cysteine. The purified cathepsin solution showed no loss of activity on keeping at 4° for several months.

Conditions of optimal activity

In order to determine the pH-activity curve, 5 ml quantities of 7.5% denatured hemoglobin in 0.005 *M* ammonium sulphate and 0.35 *M* acetic acid solution were brought to the required pH by the cautious addition of *N* hydrochloric acid or *N* sodium hydroxide while the pH was measured with a glass electrode. After suitable dilution 5 ml volumes containing 0.3 g denatured hemoglobin were incubated for 20 minutes

References p. 91.

with 1 ml purified cathepsin solution: the "colour value" (corrected for an appropriate blank) was determined as usual. The activities, expressed as "colour values", were as follows:

pH	2.8	3.0	3.4	3.8	4.2	4.4
colour value	0.18	0.73	0.79	0.80	0.70	0.54

The curve made by plotting these values indicated a pH optimum between pH 3.5 and 3.8.

Some cathepsins are known to be activated by SH-derivatives and inhibited by compounds which react with SH-groups. Compounds such as cysteine and glutathione interfered with the determination of cathepsin activity, since they reduced the Folin reagent and hence gave very high blanks. This difficulty was overcome by adding 1 ml 40% formaldehyde after each incubation to remove the excess of SH-compound. A correction was made for the effect of this addition on the "colour values".

In two series of experiments with (A) the cathepsin of human cortex and (B) a purified brain cathepsin preparation, it was found that the activity was not affected by (a) up to 0.002 *M* cysteine, (b) up to 0.0017 *M* reduced glutathione, (c) up to 0.005 *M* KCN and (d) up to 0.003 *M* iodoacetate. In agreement with these results it was found that activity of the cathepsin of human brain cortex was not affected by dialysis against distilled water overnight. It appeared that, unlike some cathepsins, the activity of brain cathepsin is not appreciably affected by SH-compounds or other dialysable activators.

Cathepsin activity in cell nuclei

Cathepsin estimations on isolated nuclei prepared from rabbit brain cortex as described by RICHTER AND HULLIN[5] gave an activity of $19 \cdot 10^{-4}$ cathepsin units/g dry weight. There may be some loss of soluble substances from the nuclei in the course of their separation and it appeared possible that activators or coenzymes might be removed. Control experiments showed however that the cathepsin activity of the nuclei was not increased by adding a concentrated extract of boiled whole brain tissue.

Figures for the cathepsin activity of the cortex and isolated nuclei of six human brains are given in Table II.

TABLE II

CATHEPTIC ACTIVITY OF HUMAN BRAIN CORTEX AND ISOLATED CELL NUCLEI

Subject	*Sex*	*Age*	*Cathepsin units/g dry weight frontal cortex* $\times 10^{-4}$	*Cathepsin units/g dry weight nuclei* $\times 10^{-4}$
D.E.	M	59	79	69
C.N.	M	63	63	47
D.R.	M	58	70	100
E.W.	M	64	66	78
F.S.	M	71	97	100
F.T.	M	50	56	70

It can be seen that the activity of the nuclear suspension was of the same order as that of the whole cortical tissue on a dry weight basis. Figures calculated for the cathepsin activity of the extra-nuclear part (mainly cytoplasm) of the tissue as described by RICHTER AND HULLIN[5] gave a mean value of 1.1 for the ratio of activities of nuclei/

References p. 91.

extra-nuclear tissue expressed in cathepsin units/g dry weight in the series of six human brains.

The cathepsin activity of the cerebral cortex of a newborn infant was found to be $37 \cdot 10^{-4}$ units/g, which is lower than the values found for adult brains.

Polypeptidase, dipeptidase and "gelatinase"

It is generally accepted that the cathepsins do not liberate amino-acids directly from proteins: this requires the presence of other enzymes which break down the polypeptides formed as intermediates. The presence of a polypeptidase in brain tissue was reported by ABDERHALDEN AND CESAR[8], who found that this enzyme was unstable at the neutral pH. The activity of the polypeptidase in rat brain tissue was readily confirmed by incubating anaerobically at 37° two suspensions (3 ml) containing (a) 250 mg rat brain suspension and (b) 250 mg brain suspension with a solution containing 184 μg peptone α-amino-nitrogen, in KREBS phosphate-Ringer pH 7.4. There was a rapid liberation of α-amino acids, amounting in 60 minutes to 84 μg α-amino-nitrogen in the tube containing peptone, above that in the control. The α-amino-nitrogen was determined by the method of MOORE AND STEIN[9]. The activity ceased after about 60 minutes owing apparently to the instability of the polypeptidase under these conditions.

The presence in brain tissue of a dipeptidase was confirmed in similar experiments in which 250 mg rat brain tissue was found to cause the liberation of 140 μg α-amino-nitrogen from an equivalent amount of glycylglycine in 60 minutes. The presence of a dipeptidase was previously reported by POPE AND ANFINSEN[10].

TAKASAKA[11] reported the presence in brain tissue of a "gelatinase" which liberates amino-acids from gelatine at pH 7.0. Attempts to confirm this failed to show any appreciable activity towards samples of purified gelatine with rat and human brain suspensions at pH 7.4 under the conditions of these experiments. EDLBACHER *et al.*[12] were also unable to confirm the existence of this enzyme in brain tissue.

DISCUSSION

The cathepsins catalyse the breakdown of proteins into polypeptides, but they do not generally liberate free amino acids: these are liberated only if polypeptidases and dipeptidases are also present. The brain cathepsin resembles in its properties a "Cathepsin I" on FRUTON AND BERGMANN's classification of proteolytic enzymes[13], since it is not activated by compounds with SH-groups; but it differs from the proteinases in this group in that the pH optimum is at pH 3.5 for proteolysis instead of in the region pH 5.4, and the partially purified enzyme is not protected from inactivation at 37° by cysteine.

Recent work with isotopically labelled amino acids has shown that the proteins of animal tissues are in equilibrium with the free amino acids in the tissues. It is believed that the proteolytic enzymes, besides being responsible for the continual breakdown of proteins, are also concerned in their resynthesis. The properties of the brain cathepsin and its presence in high concentration in the grey matter of the brain, as well as its activity in the cell nuclei are consistent with the view that this enzymes functions *in vivo* as a transpeptidase which catalyses the breakdown and resynthesis of proteins in the brain.

References p. 91.

ACKNOWLEDGEMENT

The authors thank the Medical Research Council for a grant to one of us (G.B.A.) and Prof. GOUGH for making available the human autopsy material.

SUMMARY

1. An active cathepsin is present in rat, rabbit, bovine and human brain.
2. The cathepsin is more active in the grey matter than in the white. It is active in isolated nuclei from cells of the cerebral cortex.
3. The cathepsin is stable to keeping. It acts optimally at pH 3.5 to 3.8. The activity is not effected by 0.002 *M* cysteine, glutathione, cyanide or iodoacetate.
4. The presence of a polypeptidase and a dipeptidase in brain tissue was confirmed. No evidence was found of the "gelatinase" reported by TAKASAKA.

RÉSUMÉ

1. Le cerveau de l'homme, du rat, du lapin et du bœuf, renferme une cathepsine active.
2. La cathepsine est plus active dans la matière grise que dans la matière blanche. Les noyaux isolés des cellules du cortex cérébral présentent une activité cathepsique.
3. La cathepsine est stable à la conservation. Son pH optimum est situé entre 3.5 et 3.8. L'activité n'est pas affectée par la cystéine, le glutathion, les cyanures et l'iodoacétate à des concentrations 0.002 *M*.
4. La présence d'une polypeptidase et d'une dipeptidase dans le tissu cérébral est confirmée. Les auteurs n'ont trouvé aucune preuve de l'existence de la "gélatinase" signalée par TAKASAKA.

ZUSAMMENFASSUNG

1. Ein aktives Kathepsin ist im Ratten-, Kaninchen-, Rinder- und im menschlichen Gehirn vorhanden.
2. Das Kathepsin ist in der grauen Gehirnsubstanz aktiver als in der weissen. Es ist wirksam auf die isolierten Kerne von Zellen der Grosshirnrinde.
3. Kathepsin ist stabil beim Aufbewahren. Sein pH-Optimum liegt bei pH 3.5–3.8. Die Aktivität wird nicht von 0.002 *M* Cystein, Glutathion, Cyanid oder Jodacetat beeinflusst.
4. Die Anwesenheit einer Polypeptidase und einer Dipeptidase in den Gehirngeweben wurde bestätigt. Es konnte kein Anzeichen für die von TAKASAKA berichtete "Gelatinase" gefunden werden.

REFERENCES

1 M. W. KIES AND S. SCHWIMMER, *J. Biol. Chem.*, 145 (1942) 685.
2 J. S. FRUTON, R. B. JOHNSTON AND M. FRIED, *J. Biol. Chem.*, 190 (1951) 39.
3 H. HYDÉN, *Acta physiol. scand.*, 6, Suppl. 17 (1943).
4 A. L. DOUNCE, *J. Biol. Chem.*, 147 (1943) 685.
5 D. RICHTER AND R. P. HULLIN, *Biochem. J.*, 48 (1951) 406.
6 V. ALLFREY, H. STERN AND A. E. MIRSKY, *Nature*, 169 (1952) 128.
7 M. L. ANSON, *J. gen. Physiol.*, 22 (1938/9) 79.
8 E. ABDERHALDEN AND G. CESAR, *Fermentforschung*, 16 (1940) 255.
9 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 367.
10 A. POPE AND C. B. ANFINSEN, *J. Biol. Chem.*, 173 (1948) 305.
11 T. TAKASAKA, *Biochem. Z.*, 184 (1927) 390.
12 S. EDLBACHER, E. GOLDSCHMIDT AND V. SCHLÄPPI, *Z. physiol. Chem.*, 227 (1934) 118.
13 J. S. FRUTON, G. W. IRVING AND M. BERGMANN, *J. Biol. Chem.*, 138 (1941) 249.

Received June 25th, 1953