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Protease and Ribonuclease Activities in Bovine Pituitary Lobes

By J. C. PICKUP and D. B. HOPE
Department of Pharmacology, University of Oxford, Oxford OX1 3QT, U.K.

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1. Acid and alkaline protease activities in bovine anterior and posterior pituitary lobes were reinvestigated by measurement of u.v. and Folin–Ciocalteu colour values of trichloroacetic acid-soluble digestion products of denatured haemoglobin. 2. Both lobes of the pituitary gland contain a cathepsin with a pH optimum at 3.8. 3. When release of u.v.-absorbing material was used as the assay there was also an optimum at pH 8.3–9.7, but this proved to be due to the release of nucleosides from an endogenous substrate. 4. The presence of a 'cyclizing' ribonuclease active at alkaline pH on endogenous RNA was confirmed by the inhibitory effects of phosphate, arsenate and bentonite. The activity was unaffected by heat, EDTA or metal ions. The enzyme also acted on exogenous RNA. 5. A purified preparation of neurosecretory granules from fresh bovine posterior pituitary lobes was free from alkaline ribonuclease activity. Most of the activity present in the tissue was recovered in the supernatant plus microsomal material. 6. The distribution of RNA did not follow that of the alkaline ribonuclease.

Interest in the proteolytic activity of the neurohypophysis arises from a variety of researches. On the basis of isotope studies, Sachs & Takabatake (1964) have proposed a 'precursor model' for the biosynthesis of vasopressin, in which the octapeptide is synthesized as part of a macromolecule (probably a protein) on the ribosomes of neurosecretory cell bodies. It is tempting to speculate that the hormones and neurophysins (the proteins present in the neurosecretory granules that specifically but non-covalently bind oxytocin and vasopressin) may share a common precursor. Lysis of precursor peptide bonds by a proteinase during granule maturation may result in the release of biologically active hormone, analogous, for example, to the formation of insulin from proinsulin (Grant, Coombs, Thomas & Sargent, 1971).

Dean, Hollenberg & Hope (1967) have shown that at least one of the bovine neurophysins is degraded during isolation and purification, unless precautions are taken to destroy catheptic activity. Proteolysis of neurophysins *in vitro* or *in vivo* may account, at least in part, for the extraordinary abundance of free amino acids and peptides in posterior pituitary lobes (Ramachandran & Winnick, 1957; Lande, Lerner & Upton, 1965; Preddie, 1965; Penders & Arens, 1966; Upton, Lerner & Lande, 1966; LaBella, Vivian & Queen, 1968; Schally & Barrett, 1968; Shin, LaBella & Queen, 1970; Vellan, Gjessing & Stalsberg, 1970).

It has been suggested (Shin *et al.* 1970) that secretion in posterior pituitary lobes may be controlled by fusion of lysosomes with neurosecretory granules and subsequent proteolysis of granule contents by lysosomal cathepsins, similar to the disposal of excess of secretory granules of the anterior pituitary gland (Smith & Farquhar, 1966). Holzman & Peterson (1969) have shown that exogenous protein can be taken up by mammalian neurons by endocytosis and eventually incorporated into dense and multivesicular bodies (lysosomes). It is possible that some of the newly released granular protein from the neurohypophysis may suffer a similar fate.

In the present work we have reinvestigated the acid and alkaline protease activities first described in the pituitary gland by Adams & Smith (1951), emphasizing, in particular, the identity of the so-called neurohypophysial 'alkaline protease', and with some reference to anterior-pituitary-lobe proteinase activity. A preliminary account of this work has been published (Pickup & Hope, 1971).

MATERIALS AND METHODS

Biological materials. Acetone-dried bovine posterior-pituitary-lobe powder was supplied by Paines and Byrne Ltd., Greenford, Middx., U.K.

Fresh bovine pituitary glands were obtained from the Oxford and District Co-operative Society Ltd. slaughterhouse, and were placed on ice as soon as possible after death. The anterior and the posterior lobes were separated, dissected out and weighed.

Protease assay. A modification of the procedure described by Barrett (1967) was used. An 8% (w/v) solution

of bovine haemoglobin powder [type II; Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] was made up in water and the pH adjusted to 1.8 with 1M-HCl. The solution was incubated for 1h at 45°C and then dialysed (Visking 18/32 membrane) against 5 litres of water at 4°C for 24h. The haemoglobin was then diluted to 2% with water and stored at −25°C until used.

The following buffers in the range pH1.5–11.5 were prepared and used to determine the effect of pH on proteolytic activity: sodium formate, sodium acetate, tris–HCl and glycine–NaOH (all 0.3M).

Enzyme preparations were as follows. For acetone-dried bovine posterior-pituitary-lobe powder, 1g was stirred at 4°C for 18h with 30ml of sodium acetate buffer, pH4.0 and I 0.05, and then centrifuged at 2500rev./min for 30min at 0°C in an MSE Mistral 6L refrigerated centrifuge. The supernatant was discarded and the sediment washed thoroughly with ice-cold water. Then 500mg of moist sediment was suspended in 10ml of water and samples were used in the assay. For fresh posterior pituitary glands, three lobes were dissected out (about 0.8g) and chopped to a fine mince on a Perspex board with a steel knife. The glands were homogenized in 20 times their weight of water at 0°C in a smooth-walled glass tube fitted with a Teflon pestle rotating at 950rev./min. A homogenate of bovine anterior pituitary lobes was prepared in a similar manner, from about 1.3g of tissue.

Incubation mixtures contained 0.25ml of buffer, 0.25ml of enzyme preparation and 0.5ml of haemoglobin solution. The final pH of each tube was measured with a microglass electrode. Incubation was at 37 or 45°C in a metabolic shaker (Compenstat; A. Gallenkamp and Co. Ltd.). The reaction was stopped by adding 5ml of 3% (w/v) trichloroacetic acid from an automatic pipette and the contents were mixed on a Whirlimixer (Scientific Industries Ltd.). The precipitated protein was removed by spinning at 20000rev./min for 10min in the Spinco model L refrigerated ultracentrifuge (A40 rotor). The extinction of the supernatant was measured at 280nm in a Zeiss model PMQII spectrophotometer. A 1ml sample of supernatant was withdrawn from each tube and added to 2ml of 1M-NaOH; 0.5ml of Folin–Ciocalteu reagent (1:2 dilution) was added with immediate mixing and after 30min the extinction at 750nm was measured in the spectrophotometer. Controls were obtained by adding the enzyme preparation after the trichloroacetic acid.

Examination of the degradation products. A 0.5g portion of acetone-dried posterior-pituitary-lobe powder was suspended in 15ml of water and the pH adjusted to 8.3 with 0.1M-NaOH. The suspension was incubated at 37°C for 1h, 15ml of 10% (w/v) $HClO_4$ was added and the mixture was then centrifuged at 20000rev./min for 10min in the Spinco model L ultracentrifuge (A40 rotor). The supernatant was cooled in an ice bath and 5M-K_2CO_3 was added dropwise until the solution was just alkaline (indicator paper). The white precipitate of $KClO_4$ was removed by centrifugation.

(*a*) Absorption spectrum. The supernatant was diluted 1:20 and the u.v.-absorption spectrum determined in a Unicam SP.800 spectrophotometer (silica cuvettes of 1cm path length).

(*b*) Test for deoxyribose. The method of Dische (1930) was used on a 1ml sample of supernatant.

(*c*) Test for ribose. This was carried out by using the orcinol method (Schneider, 1957) on 0.2ml of supernatant.

(*d*) Paper chromatography. The supernatant was evaporated to dryness in a rotary evaporator at 37°C and the residue was redissolved in 2ml of water. Whatman 3MM paper was cut into strips (15cm×57cm) and 0.5ml batches of supernatant were applied in a line 8cm from one short edge. The opposite edge was notched to allow solvent to run off into the bottom of the tank. The paper was eluted (descending) for 48h with propan-2-ol–water (7:3, v/v) with 0.35ml of NH_3 solution (sp.gr. 0.88) added for each litre of gas space in the tank (Markham & Smith, 1952*a*). Bands were detected in u.v. light.

The fast-running component was eluted with water and dried *in vacuo* over conc. H_2SO_4. The residue was redissolved in 0.5ml of water and incubated at 37°C for 30min with 0.1mg of bovine pancreatic ribonuclease A [5× crystallized, type 1-A; Sigma (London) Chemical Co. Ltd.]. A 0.1ml sample was spotted on to Whatman 3MM chromatography paper and eluted as before.

Testing of activators and inhibitors of alkaline ribonuclease on the activity at alkaline pH. Incubation tubes contained 0.5ml of 0.5M-tris–HCl buffer, pH8.3, and 0.5ml of enzyme preparation, which was a suspension of 100mg of bovine acetone-dried posterior-pituitary-lobe powder in 10ml of 0.5M-tris–HCl buffer, pH8.3. Activators and inhibitors were made up in the pH8.3 buffer, except for PO_4^{3-}, which was simply 0.2M-NaH_2PO_4 adjusted to pH8.3 with 4M-NaOH. The concentrations are given in the Results section.

The effect of acid and heat was assessed by suspending 100mg of acetone-dried posterior-pituitary-lobe powder in 1ml of water and adjusting the pH to 3.7 with acetic acid. The mixture was heated at 100°C for 5min, cooled and made up to 10ml with 0.5M-tris–HCl buffer, pH8.3. A 0.5ml sample was used as before.

The effect of antiserum to bovine pancreatic ribonuclease A on the activity of the alkaline enzyme was determined by using incubation mixtures of 0.5ml of antiserum (diluted 1:1) and 0.5ml of enzyme as before.

Incubation was at 37°C for 1h, after which the reaction was stopped by adding 5ml of 3% (w/v) trichloroacetic acid from an automatic pipette, followed by vigorous mixing (Whirlimixer). The precipitated protein and RNA was removed by centrifugation at 20000rev./min for 10min in the Spinco model L ultracentrifuge (A40 rotor). The E_{260} of the supernatant was measured. Blank values were obtained by adding the enzyme immediately after addition of trichloroacetic acid.

Dependence of ribonuclease activity on pH. The effect of pH on the ribonuclease activity of fresh and acetone-dried posterior pituitary lobes was studied by using a modification of the assay method described by Kalnitsky *et al.* (1959). Sodium acetate, tris–HCl and glycine–NaOH buffers (0.4 and 0.3M) were prepared in the range pH3–11. Highly polymerized yeast RNA (BDH Chemicals Ltd., Poole, Dorset, U.K.) (1mg/ml of water) was used as substrate. The enzyme was prepared from acetone-dried posterior pituitary lobes by suspending the powder (100 mg) in 15 ml of water, and from fresh glands by homogenizing (three lobes; 0.96g) in 15ml of ice-cold 0.3M-sucrose solution. Incubation tubes contained 0.25ml of buffer, 0.25ml of RNA soution and 0.5ml of enzyme preparation. Incubation was at 37°C for 10min, after which the re-

action was stopped by adding 1 ml of 0.75% (w/v) uranyl acetate in 25% (w/v) $HClO_4$ and mixing. The precipitated protein and RNA were removed by centrifugation and the supernatant diluted by adding 2.5 ml of water to a 0.2 ml sample. Controls were obtained by adding the RNA solution immediately after the uranyl acetate–$HClO_4$.

Subcellular fractionation of posterior pituitary lobes. In each experiment six glands (about 2 g of fresh tissue) were chopped to a fine mince and homogenized in 15 ml of ice-cold 0.3 M-sucrose in a smooth-walled glass tube fitted with a Teflon pestle (Kontes Glass Co.) rotating at 950 rev./min and with a radial clearance of 0.15 mm. Three upward and downward thrusts were given.

Differential centrifugation was as described by Dean & Hope (1967). Four fractions were prepared: I, sedimenting at 1100*g* for 15 min; II, sedimenting at 3900*g* for 15 min; III, sedimenting at 26000*g* for 15 min; IV, the remaining supernatant. A non-linear sucrose density gradient was prepared by layering 2.0 ml of 2.0 M-sucrose, 1.0 ml of 1.40 M-sucrose and 0.5 ml each of 1.35 and 1.30 M-sucrose over each other and equilibrating for 18 h at 4°C (Dean & Hope, 1967). A 0.5 ml portion of resuspended fraction III was layered over each of three gradients and the tubes were centrifuged at 101000*g* for 1 h in the Spinco model L ultracentrifuge (SW39 rotor). The tubes were cut with a Schuster centrifuge-tube cutter and the volume of each subfraction was measured. The density of each subfraction was measured by weighing a sample in a pre-cooled 0.2 ml constriction pipette.

Fractions were frozen and stored at −25°C until analysed.

Analytical procedures used in subcellular fractionation. (*a*) Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used.

(*b*) Determination of RNA. RNA was assayed by using a modification of the Schmidt & Tannhauser (1945) procedure. A 0.2 ml sample was mixed with 3 ml of cold 0.6 M-$HClO_4$ and allowed to stand for 15 min at 0°C. The precipitate was removed by centrifugation and washed with more cold 0.6 M-$HClO_4$, 3 ml of 0.3 M-NaOH was added to the precipitate, and the mixture was stirred and then incubated at 37°C for 1 h. The hydrolysate was acidified to pH 1 by adding 8 drops of 5 M-HCl. The solution was centrifuged and the E_{260} of the supernatant measured. As a check on protein contamination, the protein concentration in the supernatant was measured by the method of Lowry *et al.* (1951) and the E_{260} corresponding to this amount of protein was calculated from a standard curve of protein concentration against E_{260} for bovine serum albumin powder [Sigma (London) Chemical Co. Ltd.].

(*c*) Cathepsin D assay. This was carried out by using a modification of Barrett's (1967) method. Haemoglobin solution was prepared as described under 'Protease assay' above. Incubation tubes contained 0.5 ml of 1.0 M-sodium formate buffer, pH 3.8, 0.25 ml of 2% (w/v) haemoglobin solution and 0.20 ml of enzyme. Incubation was at 45°C for 1 h. Subsequent steps were as described above.

(*d*) Fumarase assay. Fumarase activity was determined by the method of Racker (1950). A 0.2 ml enzyme sample was added to 3.0 ml of 0.05 M-sodium L-malate in 0.05 M-KH_2PO_4–NaOH buffer, pH 7.4. The increase in E_{240} due to formation of fumarate at room temperature was followed against a blank which lacked the L-malate.

(*e*) Lactate dehydrogenase assay. This was carried out by the method of Wróblewski & La Due (1955). Incubations consisted of 0.1 ml of enzyme, 3 ml of 0.05 M-KH_2PO_4–NaOH buffer, pH 7.4, containing 0.31 mM-sodium pyruvate and 0.05 ml of 9 mM-NADH. The decrease in E_{340} due to the disappearance of NADH was followed at room temperature in a Unicam SP. 800 u.v. spectrophotometer against a blank containing all components except sodium pyruvate.

(*f*) Acid ribonuclease assay. Tubes contained 0.5 ml of 0.2 M-sodium acetate buffer, pH 5.5, 0.25 ml of RNA solution (1 mg/ml) and 0.20 ml of enzyme. Incubation was at 37°C for 1 h and subsequent steps were as described under 'Dependence of ribonuclease activity on pH' above.

(*g*) Alkaline ribonuclease. Tubes contained 0.5 ml of 0.2 M-glycine–NaOH buffer, pH 9.5, 0.25 ml of RNA solution (1 mg/ml) and 0.2 ml of enzyme. Incubation was at 37°C for 1 h and then as described under 'Dependence of ribonuclease activity on pH' above.

(*h*) Biological assays. Pressor activity was measured by the method of Dekanski (1952) with the modifications described by Dean & Hope (1967). Oxytocic activity was assayed on the isolated rat uterus by using the method of Holton (1948) with the Mg^{2+}-free van Dyke–Hastings solution suggested by Munsick (1960). Local standards of synthetic oxytocin and vasopressin were standardized against the IIIrd International Vasopressor, Antidiuretic and Oxytocic Standard (Bangham & Mussett, 1958). Results were calculated by using the (1+2) method of Gaddum (1959).

Immunological procedures. Antibodies against crystalline bovine pancreatic ribonuclease A were raised in rabbits by using the following method. A 2 mg portion of ribonuclease A was dissolved in 1 ml of 0.67 M-sodium phosphate buffer, pH 7.4, and 1 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.) was added. The mixture was emulsified with a serum needle fitted to a 2 ml disposable syringe. Each of two rabbits was injected with 0.95 ml (i.e. 0.95 mg) in two intramuscular sites. After 6 weeks 2.3 mg of bovine pancreatic ribonuclease A was dissolved in 1.15 ml of 0.67 M-sodium phosphate buffer, pH 7.4, and 1.15 ml of Freund's incomplete adjuvant was added. The mixture was emulsified as before and each rabbit injected with 1.15 ml in two intramuscular sites. A week later 10 ml of blood was collected from the marginal ear vein of each rabbit. The blood was clotted by incubating at 37°C for 1 h and the clot contracted by being kept at 0°C for 30 min. The contents of each tube were centrifuged and the serum was withdrawn with a Pasteur pipette. Sodium azide (10 mg) was added to each serum sample as a preservative.

The cross-reactivity of antisera was assessed by micro-immunodiffusion in 1% agar gel buffered with veronal–acetate buffer, pH 8.6 and I 0.1, containing 40 mg of Dextran 10/ml (Hellsing, 1969).

RESULTS

The effect of pH on the proteolytic activity of fresh bovine posterior pituitary lobes is shown in Fig. 1. Activity is represented by the increase in E_{280} and the colour produced by Folin–Ciocalteu

reagent with the trichloroacetic acid-soluble digestion products of denatured haemoglobin. A cathepsin with pH optimum at 3.8 is clearly demonstrated because the peak is represented by both u.v.-absorbing and Folin-positive material. An optimum at pH 9.7, however, is given by u.v.-absorbing material, which is Folin-negative and not therefore peptide or the result of proteolytic activity.

The experiment was repeated with fresh bovine anterior pituitary lobes and acetone-dried bovine posterior-pituitary-lobe powder. In the latter instance the enzyme preparation consisted of a suspension of insoluble sediment from powder extracted with sodium acetate buffer, pH 4.0 and I 0.05 (see the Materials and Methods section). We have found (J. C. Pickup & D. B. Hope, unpublished work) that 80% of the activity of cathepsin D, a lysosomal enzyme that is normally freely soluble, in acetone-dried powder from posterior pituitary glands is localized in the insoluble fraction. The proportion of the enzymic activity that is insoluble increases with the age of the powder. Both fresh anterior and acetone-dried powder from posterior pituitary lobes showed a pattern of proteolytic activity very similar to Fig. 1, with a cathepsin at pH 3.8 and a peak of u.v.-absorbing material, which was Folin-negative, at pH 9.7 (fresh anterior pituitary lobe) or pH 8.3 (acetone-dried posterior-pituitary-lobe powder).

Examination of the u.v.-absorbing material released at alkaline pH. A suspension of acetone-dried posterior-pituitary-lobe powder was adjusted to pH 8.3 and incubated at 37°C for 1h. The perchloric acid-soluble products were examined and found to have a u.v.-absorption spectrum similar to that of nucleotides, i.e. an absorption maximum at about 260nm. This indicates that the products of the alkaline enzyme are derived from an endogenous substrate (present in the powder) and not split off the haemoglobin.

Presence of sugar. The presence of ribose in the perchloric acid-soluble supernatant was shown by a green colour with the orcinol reagent. The test for deoxyribose (Dische, 1930) was negative.

Paper chromatography. A concentrate of the supernatant containing the u.v.-absorbing reaction products was applied in a band to Whatman 3MM chromatography paper, as described in the Materials and Methods section, and eluted with propan-2-ol–water–ammonia. The bands showed as dark areas when the chromatogram was irradiated with u.v. light. Fig. 2(*a*) shows, diagrammatically, the elution pattern, which was similar to that described by Markham & Smith (1952*b*) for the products of digestion of RNA by bovine pancreatic ribonuclease A. The fast-running component was eluted and

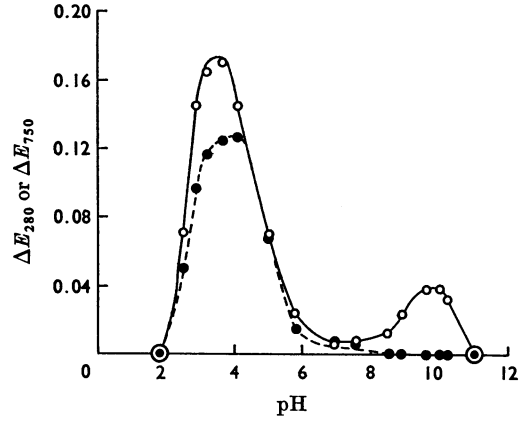

Fig. 1. Effect of pH on the proteolytic activity of bovine posterior pituitary lobes. Activity is represented as the increase in E_{280} (○) or E_{750} (Folin colour) (●) produced with the trichloroacetic acid-soluble digestion products of denatured haemoglobin. The incubation temperature was 37°C.

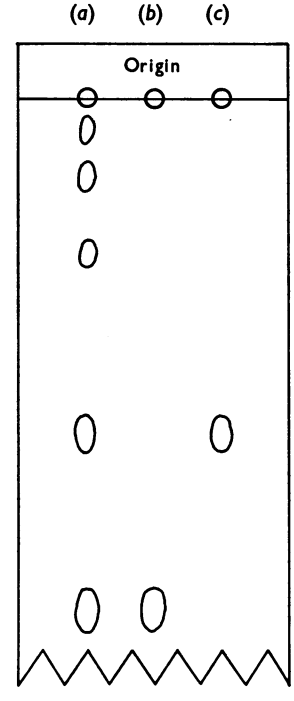

Fig. 2. Paper chromatography in propan-2-ol–water–aq. NH_3. (*a*) U.v.-absorbing material produced by the alkaline enzyme; (*b*) rechromatography of the eluted fast-running component from (*a*); (*c*) eluted fast-running component from (*a*) incubated with bovine pancreatic ribonuclease A.

when rechromatographed gave a spot with the same R_F as before (Fig. 2*b*), and when incubated with bovine pancreatic ribonuclease A at 37°C for 30min and chromatographed as before gave a slower-running spot (Fig. 2*c*), consistent with the conversion of nucleoside cyclic 2′:3′-phosphate into nucleoside 3′-phosphate (Markham & Smith, 1952*a*).

Effect of activators and inhibitors. The possibility of a 'cyclizing' ribonuclease active at alkaline pH was further tested by the effects of various substances on the activity in acetone-dried posterior-pituitary-lobe powder at pH 8.3. Table 1 summarizes the results obtained. At the concentrations tested, Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+} and EDTA produced no significant inhibition. Co^{2+} inhibited the activity slightly (73% activity remaining), but low concentrations of PO_4^{3-}, AsO_4^{3-} and iodoacetamide markedly inhibited the activity (77, 73 and 45% respectively). Bentonite completely inhibited the enzyme. L-Cysteine caused a 62% activation of the enzyme. Neither boiling at pH 3.7 nor incubation in the presence of antiserum to bovine pancreatic ribonuclease A significantly affected the alkaline enzyme activity. Bovine pancreatic ribonuclease A (final concentration 22.5 μg/ml) was inhibited by 30% when incubated with a 1:1 (v/v) dilution of the antiserum, as above.

Table 1. *Effect of substances on the activity of the alkaline enzyme*

A suspension of bovine acetone-dried posterior-pituitary-lobe powder was incubated at 37°C and pH 8.3 with addition of various activators and inhibitors.

Substance	Concn.	Activity (%)
Ca^{2+}	1mM	101
Mg^{2+}	1mM	109
Zn^{2+}	1mM	100
Co^{2+}	1mM	73
Mn^{2+}	1mM	101
EDTA	10mM	105
PO_4^{3-}	0.2M	23
AsO_4^{3-}	0.15M	27
L-Cysteine	10mM	162
Iodoacetamide	1mM	55
Bentonite	1mg/ml	0
100°C at pH 3.7	—	101
Antiserum to bovine pancreatic ribonuclease A	1:1 (v/v)	106

Ribonuclease activity with an authentic sample of RNA as substrate. The pH-dependence of the ribonuclease activity of fresh and acetone-dried posterior pituitary lobes was determined with exogenous RNA as substrate. Fig. 3 shows that there were two optima in each case, one at pH 6.9 and the other at pH 9.3 (for acetone-dried powder *I* 0.10), at pH 5.9 and 9.1 (for acetone-dried powder *I* 0.075) and at pH 5.2 and 8.0 for fresh lobes. Boiling of the powder enzyme preparation at pH 3.7 for 5min changed the position of the optima, which we have interpreted as a marked decrease in acid ribonuclease activity but little if any effect on the alkaline ribonuclease activity (Fig. 3).

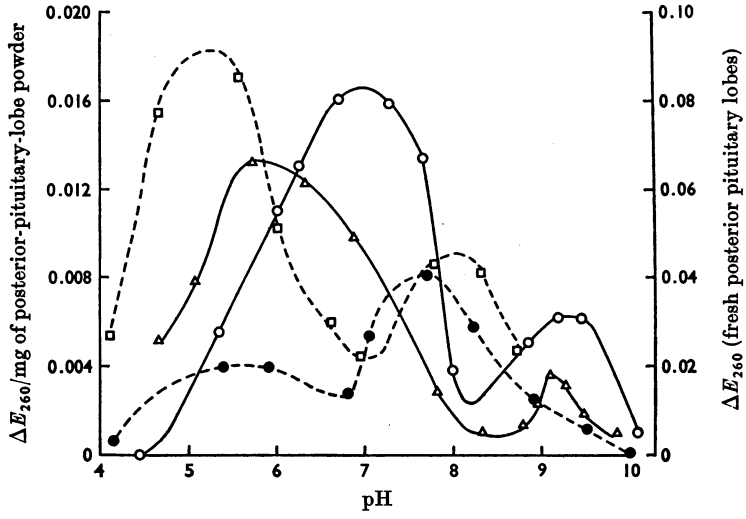

Fig. 3. Effect of pH on the ribonuclease activity of fresh and acetone-dried posterior pituitary lobes and the effect of boiling the enzyme preparation at acid pH. Exogenous yeast RNA was used as substrate. ○, Acetone-dried powder, *I* 0.1; △, acetone-dried powder, *I* 0.075; □, fresh posterior pituitary lobes; ●, acetone-dried powder boiled at pH 3.7 for 5min (*I* 0.10).

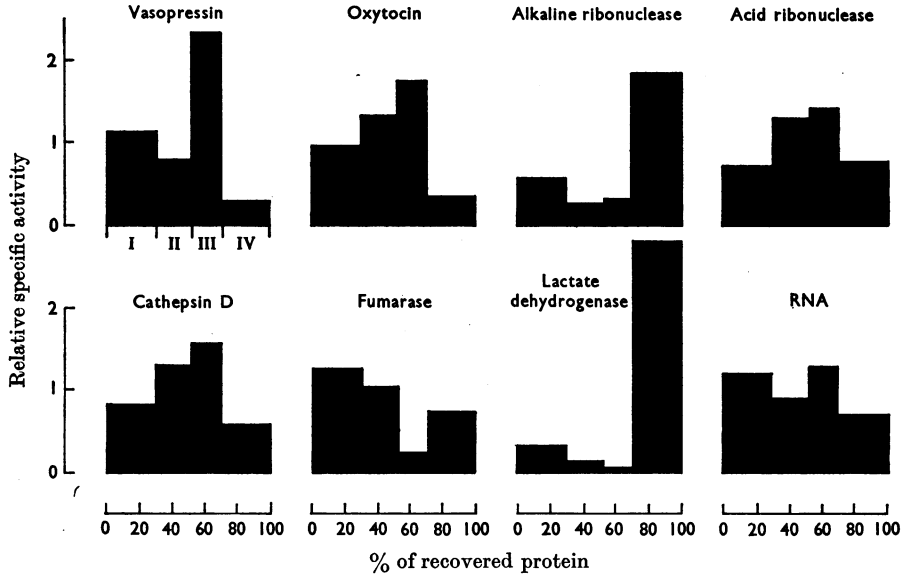

Fig. 4. Subcellular-distribution histograms of enzymic and hormonal activities and RNA in fractions obtained by differential centrifugation of homogenates of bovine posterior pituitary lobes. Results show a typical experiment. Ordinate: relative specific activity (% of recovered activity/% of recovered protein). Abscissa: % of recovered protein; left to right, fraction I (sedimenting at 1100*g* for 15min), fraction II (sedimenting at 3900*g* for 15min), fraction III (sedimenting at 26000*g* for 15min) and fraction IV (the remaining supernatant).

Subcellular fractionation of homogenates of posterior pituitary lobes. The demonstration of 'alkaline protease' activity in purified anterior-pituitary-lobe granule fractions (Perdue & McShan, 1962; Tesar, Koenig & Hughes, 1969) led us to wonder whether alkaline ribonuclease is localized in the granule fraction of homogenates of posterior pituitary lobes.

Four fractions were isolated by differential centrifugation by using a modification of the procedure of Dean & Hope (1967). Fig. 4 shows histograms of the distribution of enzymes, hormones and RNA from a typical experiment. Results are presented as relative specific activities (percentage of recovered activity/percentage of recovered protein; de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). Vasopressin and oxytocin were used as neurosecretory-granule markers, acid ribonuclease and cathepsin D as lysosomal markers, fumarase for mitochondria and lactate dehydrogenase as a cytoplasmic marker. The distribution of RNA was also studied.

It is clear that alkaline ribonuclease is almost entirely confined to the final supernatant and/or microsomal fraction (IV), which contains 72% of the recovered activity. Fraction III, which contains most of the hormonal activity (39% of the vasopressin and 29% of the oxytocin), has only 5% of the alkaline ribonuclease activity. RNA displays a notable distribution in that all four fractions have similar amounts (I, 34%; II, 23%; III, 22%; IV, 21%), contrary to the 80% or so of RNA that is found in the microsomal or supernatant fraction of most tissues (see e.g. Blobel & Potter, 1967).

RNA distribution was further studied by preparing a non-linear sucrose density gradient from 1.40M- to 2.00M-sucrose and layering 0.5ml of resuspended fraction III from the differential centrifugation on the gradient (Dean & Hope, 1967). After centrifugation the appearance of bands was as described by Dean & Hope (1967) and is shown diagrammatically in Fig. 5. Fig. 5 legend shows the volume, density and equivalent sucrose molarity for each of the six subfractions, A–F. Most of the RNA (33%) was localized in subfraction B, which also contained all of the fumarase and most of the lactate dehydrogenase activity. Neurosecretory granules, represented by oxytocin and vasopressin activities, were found mainly in fractions D and E. This indicates that RNA is associated primarily not with the neurosecretory granules but with a band containing mitochondria and some sedimentable form of cell sap.

Immunological cross-reactivity. The antiserum developed against bovine pancreatic ribonuclease A produced a single precipitin line when tested for

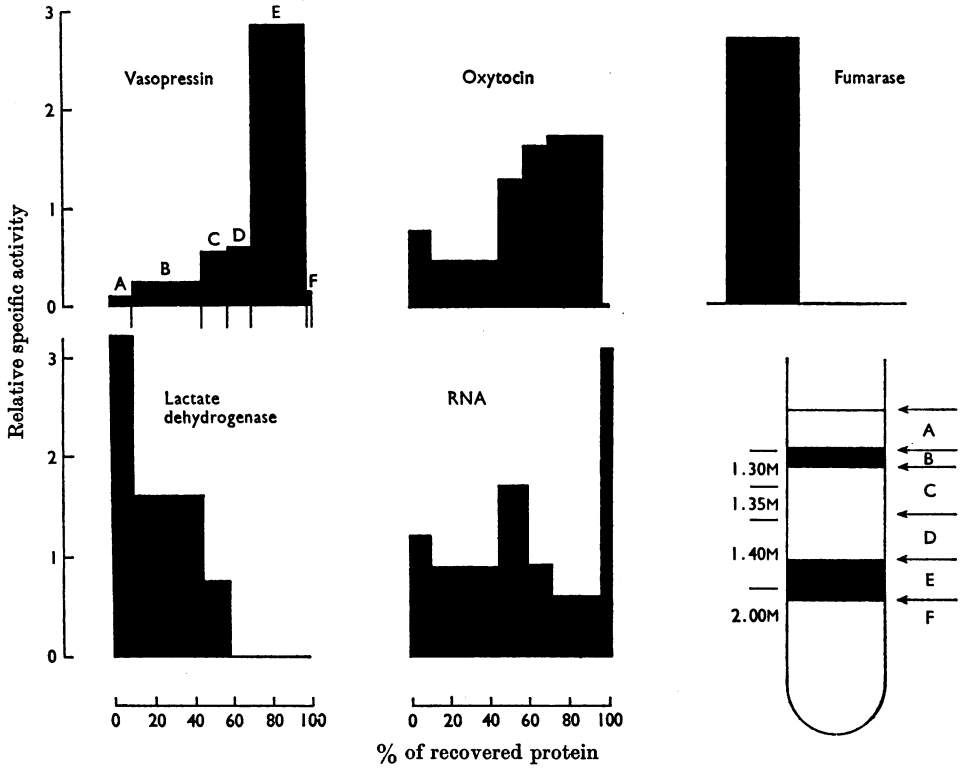

Fig. 5. Distribution histograms for enzymic and hormonal activities and RNA in subfractions from a non-linear sucrose density gradient. Fraction III from the differential centrifugation was resuspended in 0.3 M-sucrose, layered over the gradient and centrifuged at 101000*g* for 1 h. Ordinate: relative specific activity. Abscissa: % of recovered protein; left to right, subfractions A–F. The appearance of bands after centrifugation is also shown. Markings on the left of the tube indicate the concentrations of sucrose used to make the gradient. Arrows indicate the position at which the tube was sliced to obtain the six subfractions A–F, the volumes, densities and equivalent sucrose molarities of which are as follows:

Subfraction	Vol. (ml)	Density (0°C)	Molarity (20°C)
A	1.11	1.06	0.44
B	1.55	1.15	1.12
C	1.35	1.19	1.42
D	1.28	1.20	1.50
E	1.90	1.23	1.73
F	4.80	1.29	2.21

reactivity towards bovine pancreatic ribonuclease A [0.1 mg/ml in 0.85% (w/v) NaCl in 0.01 M-NaH_2PO_4 adjusted to pH 7.4 with 1 M-NaOH] by using the technique of micro-immunodiffusion. Both of the rabbits had developed antibodies against the pancreatic ribonuclease, but no precipitin lines were obtained against fraction IV from the differential centrifugation of homogenates of posterior pituitary lobes. A 5 ml portion of fraction IV was freeze-dried and redissolved in 0.5 ml of water but did not cross-react with antisera to bovine pancreatic ribonuclease A.

DISCUSSION

Acid and alkaline proteinase activities were first described in the anterior and the posterior lobes of fresh pig and ox pituitary glands by Adams & Smith (1951). These authors used an assay in which the trichloroacetic acid-soluble digestion products of denatured haemoglobin were estimated by measuring the E_{280}, a modification (Kunitz, 1947) of the original proteinase assay method of Anson (1938), which used Folin–Ciocalteu reagent to detect peptides. We have been able to confirm that

anterior and posterior pituitary lobes have cathepsic activity, but the activity at alkaline pH is associated with u.v.-absorbing material, which is Folin-negative and therefore not the result of proteolytic activity. Controls have shown that this material is not split off haemoglobin but is derived from an endogenous substrate. It is important that the controls employed by Adams & Smith (1951) and in the subsequent reports that have confirmed pituitary-gland alkaline proteinase activity by measuring E_{280} (Meyer & Clifton, 1956; LaBella & Brown, 1959; Reichert, 1961, 1962; Perdue & McShan, 1962; Tesar *et al.* 1969) did not contain the enzyme preparation alone. The need for adequate controls in protease assays because of nuclease activity has been emphasized by Marrink & Gruber (1966) and Umaña (1968). Although we could detect only one true proteinase in the anterior pituitary lobe of the ox (with a pH optimum of 3.8) it is noteworthy that Vanha-Perttula & Hopsu (1965) have described three acid and two alkaline proteinases in the rat adenohypophysis. Their assay employed Folin–Ciocalteu reagent for the detection of haemoglobin digestion products.

The identity of the alkaline ribonuclease with a 'cyclizing' enzyme has been established by a number of criteria, including u.v.-absorption spectrum and paper chromatography of the products, and the effect of various substances on the activity at alkaline pH. There are conflicting reports on the effect of metal ions and EDTA on ribonuclease activity, but most endonucleases are considered to be refractory towards these substances (Barnard, 1969). The inhibitory action of phosphate and arsenate on alkaline ribonuclease is well known (Sela & Anfinsen, 1957; Sela, Anfinsen & Harrington, 1957) and the site of action of these ions has been proposed (Kartha, Bello & Harker, 1968). Adams & Smith (1951) noted that phosphate ions inhibited the pituitary 'alkaline protease'. Bentonite is a powerful inhibitor of alkaline ribonuclease, and the enzyme is also known to be stable to boiling at acid pH (McDonald, 1948).

The neurohypophysial alkaline ribonuclease differs from other endonucleases in being activated by cysteine and inhibited by iodoacetamide. The enzyme is also unaffected by antiserum to bovine pancreatic ribonuclease A. Although many enzymes are inhibited by antibodies raised against them (Cinader, 1963), the pituitary-gland enzyme may be membrane- or ribosome-bound (see below) and inaccessible to the antibody. We have not investigated the possibiities of prolonged immunization, which increases the inhibitory power of the antiserum, probably by a decrease in specificity (Cinader, 1963). Morikawa (1967) has found that antiserum to bovine pancreatic ribonuclease A gives specific immunofluorescence with tissue sections from a number of organs, but sections of an endocrine tissue, the adrenal medulla, do not react, even though it has alkaline ribonuclease activity (see below).

It has been demonstrated that the posterior pituitary gland possesses acid and alkaline ribonuclease activities (measured with exogenous RNA). The dependence of the optima on ionic strength is like that of known ribonucleases (Kalnitsky *et al.* 1959). Because acid (lysosomal) ribonucleases are known to be acid- and heat-labile (Maver & Greco, 1962; Bernardi & Bernardi, 1966) we have interpreted the effects of boiling the posterior-pituitary-lobe powder at acid pH as a marked decrease in acid ribonuclease activity but little or no change in alkaline activity. The change in the position of the optima may reflect an increase in accessibility of the ribonuclease.

'Alkaline proteinase' activity has been demonstrated in purified anterior-pituitary-lobe granule fractions (Perdue & McShan, 1962; Tesar *et al.* 1969) and in other endocrine tissues, the adrenal gland and the thyroid gland (Todd & Trikojus, 1960). It seemed possible that alkaline ribonuclease activity would be localized in the granule fraction from homogenates of posterior pituitary lobes, especially as Philippu & Schümann (1964) have shown that adrenal-medullary granules contain ribonuclease activity. Although Smith & Winkler (1965) established that most of this activity is due to lysosomal contamination, the granules had considerable ribonuclease activity at pH 8, where the acid ribonuclease would be inactive. In our experiments, however, alkaline ribonuclease activity was localized in the supernatant and/or microsomal fraction. LaBella & Brown (1959) reported that the 'alkaline protease' of anterior pituitary lobes was also mainly found in the microsomal and supernatant fraction.

Several workers have reported that mammalian ribonucleoprotein particles and microsomes have alkaline ribonuclease activity (e.g. Tashiro, 1958; Madison & Dickman, 1963; Arora & de Lamirande, 1967, 1968). Even though it has been reported that brain ribosomes have little ribonuclease activity (Zomzely, Roberts, Gruber & Brown, 1968; Lerner & Johnson, 1970), we expected the distribution of RNA to parallel that of alkaline ribonuclease. All four fractions in the differential centrifugation, however, had similar amounts of RNA.

Reichert (1961) has found that a crude preparation of anterior-pituitary-lobe 'alkaline protease' is insoluble in many aqueous solvents, but the addition of urea increases the apparent solubility. Since the enzyme is probably alkaline ribonuclease, the effect of urea may be on the endogenous RNA substrate (Kalnitsky *et al.* 1959). If the enzyme is bound to ribosomes it may display

latency (Elson, 1959), in that intact nucleoprotein shows no ribonuclease activity, but activity appears under conditions that destroy the integrity of the ribosomes, e.g. urea. Subsequently Reichert (1962) reported that the anterior pituitary lobes from saltwater fish contain no 'alkaline protease' activity, whereas the activity in humpback-whale pituitary glands is relatively marked. These results are difficult to interpret with respect to ribonuclease.

It is generally believed that in mammalian systems alkaline ribonuclease activity is inversely proportional to the protein-synthesizing capacity of a cell (Arora & de Lamirande, 1967, 1968; Brewer, Foster & Sells, 1969), probably because of digestion of mRNA by the enzyme. In the anterior pituitary lobe, however, this would not seem to be the case, since Meyer & Clifton (1956) have found that during diethylstilboestrol-induced pituitary-gland hyperplasia and hypersecretion there was a specific increase in 'alkaline proteinase' activity.

The 'precursor model' for the biosynthesis of vasopressin (and oxytocin) (Sachs & Takabatake, 1964) suggests that a proteinase may be associated with neurosecretory granules at some point in the hypothalamo–neurophypophysial system. Osinchak (1964) has shown that newly formed neurosecretory granules in the supra-optic nucleus of the rat hypothalamus display acid phosphatase activity, and this suggests that other lysosomal enzymes, such as cathepsin, may also be present, at least in the early stages of granule formation. The present work, however, confirms that the distribution of acid proteinase does not follow that of the granules. Dean & Hope (1967) have described a procedure for the isolation of highly purified neurosecretory granules free from contamination by cathepsin (D). Although we have not been able to demonstrate neutral or alkaline protease activity in homogenates of posterior pituitary lobes, it is nevertheless possible that the granules contain a very specific proteinase that does not digest haemoglobin or an enzyme that can only be revealed by certain cofactors (Umaña, 1968).

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