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Evidence for Trypsin-like Proteases in Pituitaries of Dahl Salt Sensitive and Salt-Resistant Rats[1] (41196)

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Abstract. The pituitary cleft of Dahl hypertension-resistant rats (R) contains significant amounts of a protein-rich fluid (colloid) which is not observed in pituitary glands of Dahl salt-sensitive hypertensive (S) rats. The accumulation of colloid has been found to correlate inversely with blood pressure. In this study we have shown that the pituitaries of Dahl R rats have significantly higher levels of protease activity than salt-sensitive S rats. Protease activity was measured using the synthetic substrate α-*N*-*p*-tosyl-L-arginine-[^{3}H]methyl ester. Protease activity was found to increase with age and was highest in male R rats. The following lines of evidence suggest that colloid itself contains significant amounts of protease activity. (a) Protease activity correlated significantly with the accumulation of colloid in the R rats. (b) Protease activity could be measured directly by assaying a sample of colloid obtained by micropuncture. (c) A subline of the R rat which lacked colloid also lacked protease activity. The protease activity from R male pituitaries could be resolved into two components by Sephacryl S-200 molecular sieve chromatography. The molecular weights were estimated to be 128,000 and 64,000 daltons. Inhibitor studies showed that neither lima bean nor soybean trypsin inhibitors were potent inhibitors of protease activity. The 128,000-dalton component was more sensitive to inhibition by trasylol (Aprotinin) than the 64,000-dalton component. The role of these proteases in regulating blood pressure is not yet known.

Dahl (1) selectively bred rats for susceptibility (S strain) or resistance (R strain) to the hypertensive effect of a high salt (NaCl) diet. One of the differences that has been observed between the two strains of rats is that the pituitary clefts (Rathke's cleft) of the R rats accumulate large quantities of a protein-rich fluid (colloid) which is absent in the pituitary glands of S rats (2, 3). The term "colloid" is used in the histological context to refer to amorphous eosinophilic material. The accumulation or colloid in an F_2 population of rats derived from an S × R cross correlated inversely with blood pressure, indicating that colloid could contain a substance(s) which suppresses salt-induced hypertension (3). Standard genetic crosses showed that pituitary colloid accumulation was controlled by a single major locus (*Pc*, pituitary colloid) having two alleles, though effects of genetic background on the expression of these alleles were evident (4).

A number of unique proteins in pituitary colloid from R rats were observed by gel electrophoresis. These proteins were called R proteins and designated R1, R2, R3, and R4 in order of decreasing electrophoretic mobility. These colloid proteins have been characterized with respect to their molecular weights and isoelectric points (3). It has recently been shown that R1 and R2 are fragments of rat serum albumin (5).

In the present study pituitary protease activity was evaluated using the artificial substrate tosyl-arginine-*O*-methyl ester (TAME). TAME has been used to measure many tryspin-like proteases. This activity was found to correlate well with pituitary colloid accumulation and therefore with the accumulation of the albumin fragments contained in colloid. Some biochemical properties of the pituitary proteases are also given.

Materials and Methods. Stocks of R rats were obtained from Dr. Lewis Dahl of

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Brookhaven National Laboratory in 1972. The S rats were obtained in 1975. Lines of R rats have been inbred by us. One of the R sublines (R/A61) shows marked pituitary colloid accumulation and another R subline (R/C3) resembles the S strain in lacking pituitary colloid accumulation (4). All rats were kept on standard rat chow (Wayne Lab Blox) which contained 1% NaCl.

Rats were killed by rapid decapitation. The pituitaries were removed and graded semiquantitatively for colloid accumulation which is easily visible in the gross specimen. Grades were 0 = no colloid, 1 = slight, 2 = moderate, 3 = marked, and 4 = massive. Such grades correlate well with the accumulation of albumin fragments observed in the pituitary by gel electrophoresis (2).

Pituitaries were homogenized in a glass–glass homogenizer which contained 20 μl of 0.2 *M* Tris–HCl buffer (pH 8.0)/mg of pituitary tissue. The homogenate was centrifuged at 1000 *g* for 10 minutes at 4°. The pellet was discarded and the supernatant was assayed for serine protease activity.

Pituitary protease activity was measured using the substrate α-*N*-*p*-tosyl-L-arginine-[^{3}H]methyl ester ([^{3}H]TAME), specific activity 213 mCi/mmole (Amersham/Searle), which was purified before use (6). The assay was performed by the method of Margolius *et al.* (7) and was linear as a function of time for 1 hr and as a function of protein. A typical assay contained 10 μl of [^{3}H]TAME, 10 μl of 0.2 *M* Tris–HCl (pH 8.0) and 30 μl of test sample. Activity is expressed as cpm of [^{3}H]methanol formed per 30 minutes or per hour.

A comparison of S and R pituitary protease patterns by gel filtration chromatography was made using a calibrated Sephacryl S-200 column. The column was calibrated using the following standards: bovine serum albumin (M_r 67,000), ovalbumin (M_r 45,000), chymotrypsinogen A (M_r 25,000), and cytochrome *C* (M_r 12,400). Anesthetized rats were perfused via the left ventricle with saline. Pituitaries were devoid of visible blood following perfusion. Pituitaries from 15 S or 15 R males were homogenized in 1 ml of 0.1 *M* Tris–HCl, pH 8.0, and centrifuged for 10 min at 10,000 *g*. The supernatant was applied to a 1.5 × 90-cm Sephacryl S-200 column. The sample was eluted with a 0.05 *M* Tris–HCl buffer, pH 8.0, which also contained 0.05 *M* NaCl and 0.02% NaN_3. Two-milliliter fractions were collected and assayed for TAME esterase activity.

Micropuncture samples of pituitary cleft colloid which were free of blood were obtained from male rat pituitaries as previously described (3).

Results. Pituitary protease activity was examined in 4-week-old S and R rat pituitaries, a time when colloid accumulation is low in R pituitaries. Table I illustrates that only the R/A61 male pituitaries contain significantly elevated levels of pituitary protease activity and colloid at that age. TAME esterase activity was expressed per milligram of tissue rather than per milligram of protein because colloid contains a large amount of protein which is not really part of the intracellular protein content of the pituitary.

At 12 weeks of age pituitary protease activity increased in both S and R/A61 rats (Table II). However, male and female R/A61 pituitaries contained significantly higher levels of activity, roughly two- to threefold higher, than their S counterparts. Both male and female R/A61 pituitaries also showed significant accumulation of colloid at 12 weeks of age compared to S rat pituitaries which showed no accumulation. The increase in protease activity appears to correspond with the increased accumulation of colloid. To further illustrate this point, individual R/A61 pituitaries were graded in the intact gross specimen for colloid accumulation and this grade was plotted as a function of their pituitary protease activity (Fig. 1). A straight line was generated with a slope significantly different from zero ($P < 0.005$) demonstrating a significant relationship betwen colloid accumulation and protease activity.

Further proof that the pituitary protease activity was primarily found in colloid came from studies which compared protease activity in a subline of the R rats (R/C3) which did not contain colloid (4) (Table III). The

TABLE I. MEANS, STANDARD ERRORS, AND RESULTS OF ANALYSIS OF VARIANCE FOR PITUITARY WEIGHT AND PITUITARY PROTEASE ACTIVITY IN 4-WEEK-OLD R/A61 AND S RATS[a]

	R/A61		S		P Significance levels		
	Female (6)	Male (6)	Female (6)	Male (6)	Strain	Sex	Interaction
[^{3}H]TAME hydrolyzed, cpm/30 min/ mg of tissue	654 ± 158	1660 ± 233	502 ± 46	792 ± 97	<0.005	<0.005	0.025–0.05
Pituitary wt., mg	4.48 ± 0.27	5.83 ± 0.34	3.71 ± 0.20	4.45 ± 0.17	<0.005	<0.005	NS
Mean colloid grade	Trace	0.6	0	0			

[a] Values in parentheses indicate sample sizes. Probabilities were obtained from a two-way analyses of variance. NS, not significant (i.e., $P > 0.05$).

R/A61 rats at 16 weeks of age had significantly higher colloid levels and protease activity than the pituitaries of the R/C3 subline. In fact, the pituitaries of the R/C3 line appeared to have less protease activity than the S pituitaries (Table II).

When pure collid material was removed from intact R/A61 pituitaries by micropuncture (usually 1 to 2 μl) and assayed for TAME esterase activity, the activity of the sample was well over the linear range of the assay. Thus, colloid contains enormous amounts of protease activity and must contain the enzyme(s) responsible for the activity differences between the S and R pituitaries. Quantitation of protease activity in colloid is difficult to assess at this time because of the questionable ability to quantitatively remove colloid from Rathke's cleft.

A theoretical possibility for the lower activity of pituitary proteases in the S rat is that the S pituitary could contain protease inhibitors. To test this possibility, increasing amounts of S male pituitary cytosol were added to a constant amount of R male

TABLE II. MEANS, STANDARD ERRORS, AND RESULTS OF ANALYSIS OF VARIANCE FOR PITUITARY WEIGHT AND PITUITARY PROTEASE ACTIVITY IN 12-WEEK-OLD R/A61 AND S RATS[a]

	R/A61		S		P Significance levels		
	Female (6)	Male (6)	Female (6)	Male (6)	Strain	Sex	Interaction
[^{3}H]TAME hydrolyzed, cpm/30 min/ mg of tissue	2611 ± 467	4823 ± 1065	1568 ± 136	1382 ± 130	<0.005	NS	0.05–0.10
Pituitary wt., mg	11.8 ± 0.74	12.2 ± 0.85	14.0 ± 0.55	11.7 ± 0.30	NS	NS	0.05–0.10
Mean gross colloid grade	1.58	2.58	0	0			

[a] Values in parentheses indicate sample sizes. Probabilities were obtained from a two-way analyses of variance. NS, not significant (i.e., $P > 0.05$).

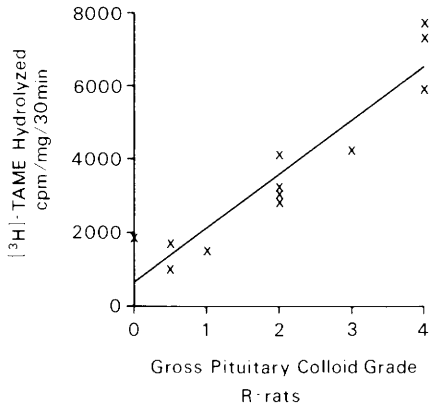

FIG. 1. Correlation of TAME esterase activity with colloid grade. Pituitary esterase activity was determined in six male and six female R rats as described under Materials and Methods. Activity is expressed as cpm of [^{3}H]methanol formed/mg of pituitary per 60-min incubation at room temperature. Colloid accumulation of each pituitary was grossly graded following removal from the animal prior to homogenization. The slope of the regression line was insignificantly different from zero ($P < 0.005$). The line was drawn according to the equation $y = 1469x + 656$. The correlation coefficient $r = 0.936$.

pituitary protease activity. The S pituitary cytosol failed to have any inhibitory effect, indicating that inhibitors are probably not involved in suppressing TAME esterase activity in S pituitaries (data not shown).

Some biochemical properties of the pituitary protease(s) were examined. Pituitary extracts from perfused S and R males were chromatographed on a calibrated Sephacryl S-200 column (1.5 × 90 cm) to estimate molecular weights of the pituitary proteases. As shown in Fig. 2 the R/A61 male pattern shows two peaks of activity corresponding to molecular weights of 128,000 ± SE of 5600 ($n = 3$) and 64,000 ± SE of 3000 ($n = 3$) for peaks 1 and 2, respectively. The S male pattern showed a major peak of activity corresponding with the R/A61 male peak 1, with a molecular weight of 137,000 ± SE of 5300 ($n = 3$). Occasionally, a second peak of protease activity was detected when S male pituitary estracts were chromatographed (data not shown in Fig. 2). Its molecular weight corresponded with peak 2 found in the R male extracts. Chromatography of colloid obtained by micropuncture of 10 R rat pituitaries on the calibrated Sephacryl S-200 column produced a pattern of [^{3}H]TAME esterase activity similar to the pattern shown in Figure 1. This is further evidence that a large portion of these proteases reside within colloid.

The molecular weight determinations of the two esterase peaks was very reproducible. Bovine serum albumin dimer, which is found in most preparations of albumin, was also used as a molecular weight marker (M_r 134,000) but was not used to estimate the molecular weight calibration line. Albumin dimer fell on the linear portion of the calibration curve indicating that estimation of

TABLE III. MEANS, STANDARD ERRORS, AND RESULTS OF ANALYSIS OF VARIANCE FOR PITUITARY WEIGHT AND PITUITARY PROTEASE ACTIVITY IN 16-WEEK-OLD R/A61 AND R/C3 RATS[a]

	R/A61		R/C3		*P* Significance levels		
	Female (5)	Male (5)	Female (5)	Male (5)	Line	Sex	Interaction
[^{3}H]TAME hydrolyzed, cpm/30 min/ mg of tissue	1402 ± 217	4223 ± 2062	491 ± 118	171 ± 30	<0.001*	NS	0.025–0.05*
Pituitary wt., mg	16.1 ± 1.0	19.4 ± 0.92	14.4 ± 0.48	15.7 ± 0.19	<0.005	0.005–0.01	NS
Mean colloid grade	0.9	3.0	0	0			

[a] Values in parentheses indicate sample sizes. Probabilities were obtained from a two-way analyses of variance. NS, not significant (i.e., $P > 0.05$).

* Analysis performed on log transformed data.

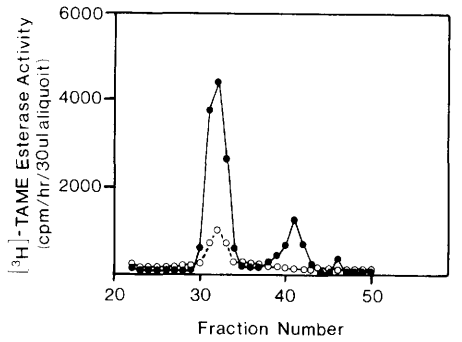

FIG. 2. Sephacryl S-200 patterns of pituitary esterase activity. Anesthetized rats were perfused via the left ventricle with saline. The pituitaries were devoid of blood following this perfusion. Pituitaries from 15 S males (open circles) and 15 R males (closed circles) were homogenized in 1 ml of 0.1 *M* Tris–HCl buffer, pH 8.0, and centrifuged for 10 min at 10,000*g*. The supernatant was applied to a (1.5 × 90-cm) calibrated Sephacryl S-200 column. The column was eluted with 0.05 *M* Tris–HCl buffer pH 8.0, which contained 0.05 *M* NaCl and 0.02% NaN_3. Two-milliliter fractions were assayed for TAME esterase activity (a 30-μl aliquot of each fraction) for 1 hr at room temperature. Activity eluting in fraction 32 was designated as peak 1. Activity eluting in fraction 41 was designated as peak 2.

molecular weights up to 130,000 daltons is reasonable. Bovine γ-globulin, however, was not on the linear portion of the curve.

Chromatography of pituitary homogenate on DEAE-Sephadex using a starting buffer of 0.01 *M* potassium phosphate buffer, pH 7.0, and eluting the column with a linear gradient of KCl from 0 to 0.7 *M* KCl resulted in the elution of major peak of esterase activity which coeluted with most of the proteins bound to the column. A minor active component eluted as a shoulder of the major peak at a slightly lower KCl concentration. Thus, the two active fractions which separate by molecular sieve chromatography cannot be resolved by ion-exchange chromatography under the conditions tested.

Inhibitor studies were performed to further characterize the nature of the active sites of the two esterase activities eluted from the Sephacryl S-200 column. Lima bean trypsin inhibitor and soybean trypsin inhibitor demonstrated only weak inhibitory activity (less than 10%) using both pituitary esterase peaks 1 and 2, isolated from male R/A61 pituitaries, up to inhibitor concentrations of 166.6 μg/ml. Trasylol (Aprotinin) inhibited the high-molecular-weight esterase activity by 50% at a concentration of 16.6 μg/ml while the low-molecular-weight esterase was only slightly inhibited by the same concentration of trasylol (22% inhibition). Thus, these two pituitary TAME esterases are trypsin like with respect to their esterase activity using TAME but are not inhibited by soybean and lima bean trypsin inhibitors.

Discussion. The large amount of trypsin-like (TAME esterase) activity in the pituitaries of R/A61 rats is contained in the pituitary colloid of Rathke's cleft. This was shown by (a) direct collection of colloid containing esterase activity, (b) correlation of colloid accumulation in R/A61 pituitaries with protease activity, and (c) a comparison of two sublines of R rats, one of which had pituitary colloid and high protease activity and the other which lacked both characteristics.

The mechanism for the accumulation of colloid remains obscure as does its role as a regulator of blood pressure. Genetic studies indicated that colloid could play a role in suppressing blood pressure (3, 4). The TAME esterases in colloid may be proteases involved in the processing of peptide hormones in the pituitary. Since colloid is accumulated in Rathke's cleft, it is in juxtaposition to cells of both the anterior and intermediate lobes of the pituitary gland, the proteases might originate from either lobe. The fact that the S and R/C3 rat pituitaries had low levels of TAME esterase activity suggests that the esterase activity may be of pituitary origin. Kenessey *et al.* (8) have found that trypsin-like protease activity in the anterior pituitary of the rat was suppressed by dexamethasone and enhanced by adrenalectomy and ovariectomy. The trypsin-like enzyme in their studies also demonstrated activity against β-lipotropin. Thus, their pituitary protease appears to be hormonally regulated and may be involved in peptide hormone processing.

Further purification of our TAME es-

terase activity will be required before hormone processing activity will be tested. With the knowledge that trasylol is an effective inhibitor of the high-molecular-weight esterase a trasylol affinity column may be useful for purifying large quantities of this esterase for future testing.

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