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THE INFLUENCE OF ANDROGENS ON ENZYMES (CHYMOTRYPSIN- AND TRYPSIN-LIKE PROTEASES, RENIN, KALLIKREIN AND AMYLASE) AND ON CELLULAR STRUCTURE OF THE MOUSE SUBMAXILLARY GLAND

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SUMMARY

1. The effect of age and androgen level on enzyme activity and cellular structure has been determined in the mouse submaxillary gland.

2. A new protease which resembles chymotrypsin in its substrate specificity has been characterized in the gland.

3. Activity of the chymotrypsin- and trypsin-like proteases and renin increased considerably in male mice concomitantly with proliferation of granules in the secretory tubules of the gland.

4. The androgen dependence of the chymotrypsin- and trypsin-like enzymes, renin and the organelles within the secretory tubules was confirmed in castrated male mice. The activity of these enzymes increased and correlated with the appearance of intracellular granules in the secretory tubules when the castrated male mice and in addition female mice were treated with testosterone preparations.

5. Kallikrein, a closely related protease, and amylase increased in activity with age but showed no sex-linked differences.

6. The results suggest that kallikrein is sequestered in acinar cells whereas the androgen-dependent enzymes (chymotrypsin, trypsin and renin) are located in the secretory tubules.

INTRODUCTION

The androgen dependence of proteolytic enzymes and cell structure within the mouse submaxillary gland has been the subject of much study. Sex-linked differences in the structure of the gland were first demonstrated by Lacassagne (1940*a*, *b*, *c*). Parallelism between the androgen-dependent increase in proteolytic activity and proliferation of secretory tubules was

pointed out by Junqueira, Fajer, Rabinovitch & Frankenthal (1949). In these early studies protease activity was determined using the non-specific substrate casein. More recently renin and, with the availability of synthetic enzyme substrates, a trypsin-like enzyme have been characterized in the mouse submaxillary gland and shown to be testosterone-dependent (Oliver & Gross, 1967; Angeletti, Angeletti & Calissano, 1967).

Similar proteases have also been reported in the rat. Initially Sreebny (1960) demonstrated an enzyme which hydrolysed both casein and benzoyl-1-arginine amide. Subsequently three trypsin-like proteases (*glandulain*, *salivain* and β-cathepsin) and four kallikrein isoenzymes were isolated from the rat submaxillary gland (Riekkinen, Ekfors & Hopsu, 1966; Riekkinen, Ekfors & Hopsu-Havu, 1967; Ekfors, Riekkinen, Malmiharju & Hopsu-Havu, 1967). Of these only *glandulain* was stimulated by testosterone (Riekkinen & Niemi, 1968).

Although the renin and trypsin-like enzymes are regulated by testosterone it is not known whether such an action is selective or whether other submaxillary secretory enzymes like kallikrein and amylase are similarly influenced. Furthermore, these secretory enzymes are stored in intracellular organelles (Bhoola & Ogle, 1966; Chang, Erdös, Miwa, Tague & Coalson, 1968) in gland tissue which contains a heterogeneous population of cells. The correlation therefore of changes in cellular structure with changes in levels of specific enzymes could provide valuable information in determining the precise cellular and subcellular location of the secretory enzymes.

In the present report we describe the effect of age and androgens on the activity of a number of secretory enzymes and on the gland morphology. The experiments were designed to compare enzyme activity and cellular structure (*a*) in males and females of the same age group, (*b*) in males after castration and the subsequent replacement of testosterone, and (*c*) in females treated with testosterone.

Some of the preliminary results have been communicated to the Physiological Society (Bhoola & Dorey, 1972).

METHODS

Mice of MRC strain L.A.C.G. were bred to enable precise age dating of the animals. For each male and female group in the age distribution study about 100 pooled glands were utilized; however, for the very early and late age groups between 200 and 400 glands were used. In the castration and testosterone injection experiments, because of the requirement for animals of similar age, it was only feasible to use eighteen to forty glands for each set of control and experimental groups.

Males, mainly 30–31 days old, were castrated under pentobarbitone sodium anaesthesia (8 mg/100 g body wt.). Sham operations were performed under similar conditions. Each hormone preparation was injected subcutaneously in a volume of

0·1 ml. after appropriate dilution in arachis oil. The long-acting oenanthate ester of testosterone, which was administered at 4 or 7 day intervals, was chosen in order to avoid the necessity for daily injections. The short-acting propionate ester of testosterone was administered daily; this preparation was included because it has been used to examine the anti-androgen effects of cyproterone acetate in previous studies.

Preparation of tissue extracts

The submaxillary glands were removed from the mice under chloroform anaesthesia, washed in 0·9 % NaCl to remove traces of blood, and freeze-dried. The pooled, freeze-dried glands were extracted with 0·067 M Tris-HCl buffer, pH 8·0. For the measurement of amylase activity, the tissue was extracted with Sörensen's buffer, pH 6·9. The tissue extract was centrifuged at 7000 *g* for 10 min; the pellet was re-extracted and the pooled supernatant used for the enzyme assay. Enzyme activity was determined in general on two, but sometimes on three, separate extracts (25 mg pooled, freeze-dried tissue/ml.) of each control and experimental group and a total of three to nineteen measurements were made on each group.

Measurement of enzymes

Estero-proteases

(i) *Chymotrypsin* (*E.C.* 3.4.4.5). The chymotrypsins are most effective in cleaving peptide or ester linkages involving tyrosine, phenylalanine and tryptophan. The esterase activity of chymotrypsin was measured on acetyl-1-tyrosine ethyl ester (ATEe) using the enzymic ethanol assay method of Trautschold & Werle (1961), which was adapted for this enzyme and substrate (C. W. Jones, unpublished results). This method proved to be about ten times more sensitive than the standard procedure using ATEe alone (Schwert & Takaneka, 1955). The assay medium for measuring ATEe activity consisted of 0·2 ml. substrate (12 m-mole ATEe), 0·1 ml. (3 m-mole) nicotinamide adenine nucleotide and 0·02 ml. (150 units) yeast alcohol dehydrogenase; buffer solution (0·15 M semicarbazide hydrochloride, 0·15 M tetrasodium pyrophosphate and 0·044 M glycine, pH adjusted to 8·4 with 2 N-NaOH) and tissue extract were added to give a final volume of 3·02 ml. The reference cell contained 0·2 ml. substrate and 2·8 ml. buffer solution. The increase in absorbance was followed at 366 nm. Because of the relative lack of specificity with ATEe as substrate (Table 1), the presence of the chymotrypsin-like enzyme in the tissue extracts was confirmed using the more specific, chromogenic substrate carboxypropionyl phenylalanine *p*-nitroanilide (CPPN). The assay medium consisted of 0·3 ml. CPPN (0·16 m-mole), Tris-HCl buffer (0·5 M Tris-HCl and 0·66 M $CaCl_2$, pH 7·5) and tissue extract, to give a cuvette volume of 3·0 ml. The *p*-nitroaniline released produced a linear increase in absorbance at 405 nm.

(ii) *Trypsin* (*E.C.* 3.4.4.1). Peptide and ester linkages involving arginine and lysine are readily hydrolysed by trypsin. The relatively specific amide substrate benzoyl-1-arginine *p*-nitroanilide (BAPA) was used to characterize the trypsin-like enzyme. The assay medium and conditions have been described previously (Bhoola & Dorey, 1971*a*).

(iii) *Kallikrein* (*E.C.* 3.4.4.21). Although kallikreins cleave mainly arginine esters such as benzoyl-1-arginine ethyl ester (BAEe), this property could not be used to identify them in the tissue extracts because of the substantial arginine esterase activity of trypsin and to a much lesser extent chymotrypsin (Table 1). In contrast, because the kininogenase (proteolytic) activity shows absolute specificity, kallikrein was measured by its ability to liberate kinins from a plasma-substrate. The released kinins were assayed on the cat jejunum against synthetic bradykinin (Bhoola & Dorey, 1971*a*).

(iv) *Total esterase activity.* An estimate of the combined esterase activity of kallikrein, trypsin and chymotrypsin was obtained with BAEe. The BAEe hydrolysing activity was measured using the alcohol dehydrogenase linked assay (Bhoola & Dorey, 1971*a*), similar to that used for ATEe.

Renin

The proteolytic activity of renin also shows a high degree of specificity. Renin activity was measured by its ability to cleave angiotensin from a plasma substrate. To determine renin activity in the gland extracts a 0·2 ml. test sample was incubated at 37° C with 0·1 ml. 3 % neomycin sulphate and 1·4 ml. plasma substrate prepared from nephrectomized rats (Oliver & Gross, 1967). After termination of the reaction the incubate was centrifuged and the supernatant assayed for released angiotensin on the rat blood pressure. The pressor activity was determined in male Wistar rats anaesthetized with urethane (v/w, 0·4 ml. (25 % solution)/100 g body wt.). The rats were vagotomized and treated with *dibenyline* (0·1 mg I.V./100 g) and pentolinium (0·5 mg I.V.). The pressor responses obtained with the test samples were bracket assayed against angiotensin II (valine^5-angiotensin II aspartic β-amide, CIBA). In control experiments it was established that the incubate (plasma substrate and tissue extract) was free of angiotensinase activity and that no spontaneous release of angiotensin occurred during the incubation.

Amylase

The iodine titration method was used to determine amylase activity (Bhoola & Heap, 1970).

Protein

Protein was measured by the method described by Lowry, Rosebrough, Farr & Randall (1951).

Expression of enzyme activity

In the case of enzyme activity measured on synthetic substrates (ATEe, BAEe, CPPN and BAPA), the rate of hydrolysis of the substrate was followed for 5 min at 25° C in a Pye-Unicam SP 1800 or 800 spectrophotometer. The linear phase of the change in absorbance was used to calculate activity which was expressed as increase in optical density units (Δ_E at 366 or 405 nm) per min per mg protein. The activity of kallikrein was expressed as μg bradykinin equivalents per mg protein and that of renin as μg angiotensin II equivalents per min per mg protein. Amylase activity was calculated as milliunits (m-u.) per mg protein.

Morphology

For the ultrastructural study the glands were fixed first in a mixture of 4 % paraformaldehyde and 2·5 % glutaraldehyde in cacodylate buffer (pH 7·3) and subsequently in 1 % osmium tetroxide in cacodylate buffer (pH 7·3). The tissue was dehydrated through the usual alcohol series and propylene oxide, and embedded in Araldite (CIBA). Sections were cut using an L.K.B. Ultrotome I. Thin sections were stained with uranyl acetate and lead citrate and observed through a Hitachi HU-11B electron microscope. Thick sections (1 μm) were stained with 1 % toluidine blue–1 % borax solution and examined by light microscopy.

RESULTS

Enzyme activity

Age- and sex-related differences

Extracts prepared from glands of 2- to 5-day-old mice showed an ATEe (chymotrypsin-like) activity of $0{\cdot}034 \pm 0{\cdot}005$, $n = 3$ (Δ_E/min.mg protein). By 30–40 days in the male the activity had increased substantially to $5{\cdot}83 \pm 0{\cdot}39$ (S.E. of mean), $n = 10$. This rapid rise in activity reached a peak between 41 and 50 days falling subsequently in the fully mature mice (Text-fig. 1). Comparable results were obtained with the more specific substrate, CPPN (Text-fig. 1). The trypsin-like enzyme measured on BAPA showed a similar age- and sex-related profile (Text-fig. 2). The distribution pattern of renin (Text-fig. 3) was comparable to that obtained with the chymotrypsin- and trypsin-like proteases. All three enzymes showed a marked sexual dimorphism.

TABLE 1. Relative potency of chymotrypsin, trypsin, kallikrein and renin on synthetic substrates

	Substrate			
Enzyme	+ATEe	+BAEe	*CPPN	*BAPA
Chymotrypsin	355	1·3	0·845	0·0012
Trypsin	6·9	136	0·0048	8·294
Kallikrein	7·4	174	0	0·013
Renin	0	0	0	0

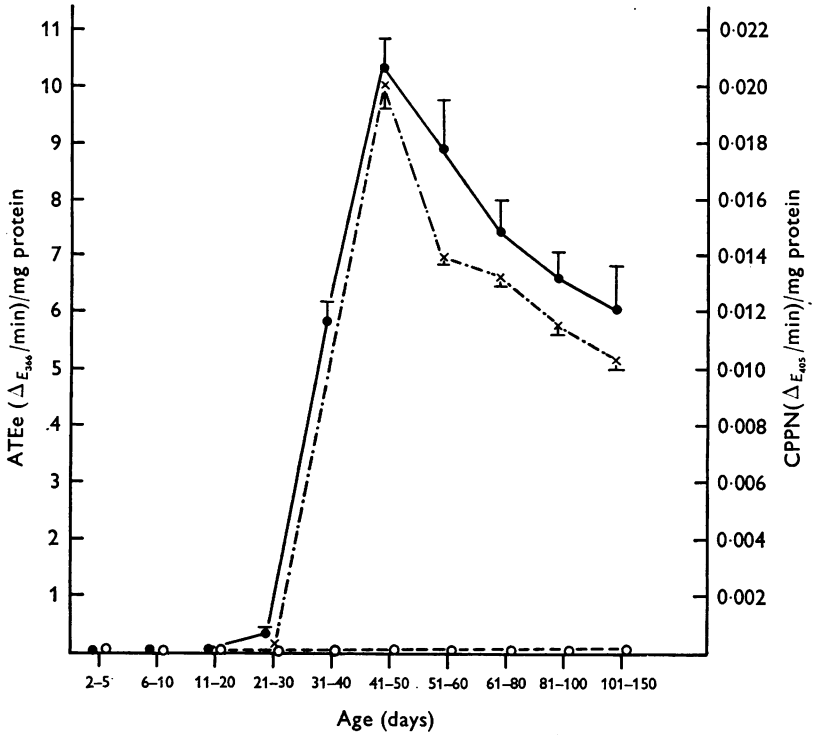
Enzyme activity expressed as Δ_E/min at 340 nm + or 405 nm* and values calculated for a cuvette enzyme concentration of 1 mg/ml.

Chymotrypsin: 3 × crystallized, lyophilized preparation from bovine pancreas (Koch Light). Not blocked for contaminating tryptic activity. Trypsin: 2 × crystallized, lyophilized preparation from bovine pancreas (Koch Light). Not blocked to exclude residual chymotryptic activity. Kallikrein: highly purified, hog submaxillary kallikrein was kindly supplied by Professor E. Werle. Renin: purified, hog kidney renin preparation (Miles Serevac).

The submaxillary glands of newborn mice contained virtually no kallikrein. The kininogenase activity began to increase 11–20 days postnatally, with a dramatic rise in activity between 21 and 30 days (Text-fig. 4). Although kallikrein activity in the female did not illustrate the marked sexual dimorphism of the androgen-sensitive enzymes, it seemed to trail slightly below that of the male. A somewhat similar profile was demonstrated for amylase. Up to 10 days after birth no amylase could be detected. Subsequently, activity appeared in the gland (11–20 days: male 37 m-u./mg protein, $n = 3$; female 22 m-u./mg protein, $n = 3$) but increased to values which showed no substantial difference between male

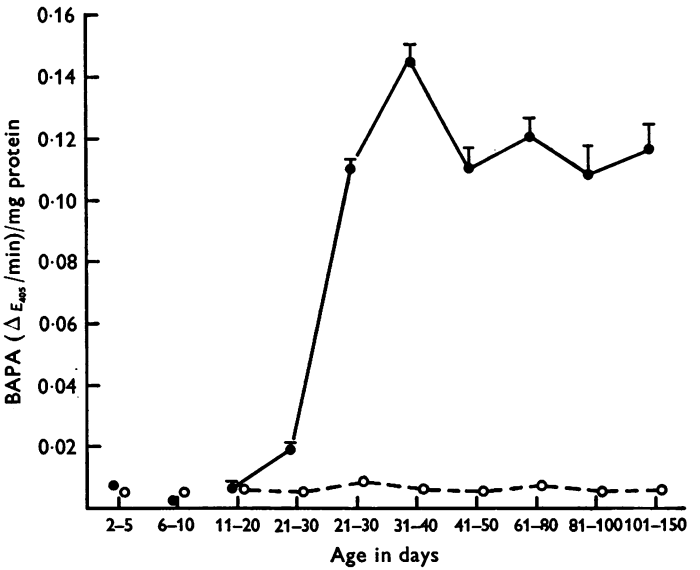
and female (100–150 days: male 82 m-u./mg protein, $n = 3$; female 97 m-u./mg protein, $n = 3$).

Each of the three estero-proteases identified in the gland extracts hydrolyse BAEe (Table 1). The BAEe activity was similar in the male and female up to 11–20 days. Thereafter, the activity in the male increased more rapidly and was much greater than that in the female (Text-fig. 5).

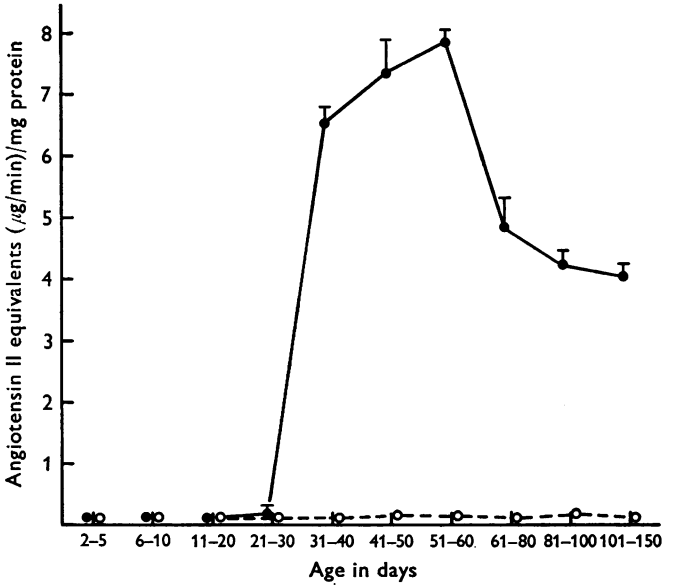


Text-fig. 1. Age- and sex-related differences in the activity of the chymotrypsin-like enzyme. Values, ATEe (male ●——●, female ○- - -○) and CPPN (male ×-·-×, female ○- - -○), are expressed as specific activity and represent mean measurements ($n = 3$–7) of separate extracts of pooled, freeze-dried submaxillary glands. Bar shows 1 S.E. of mean.

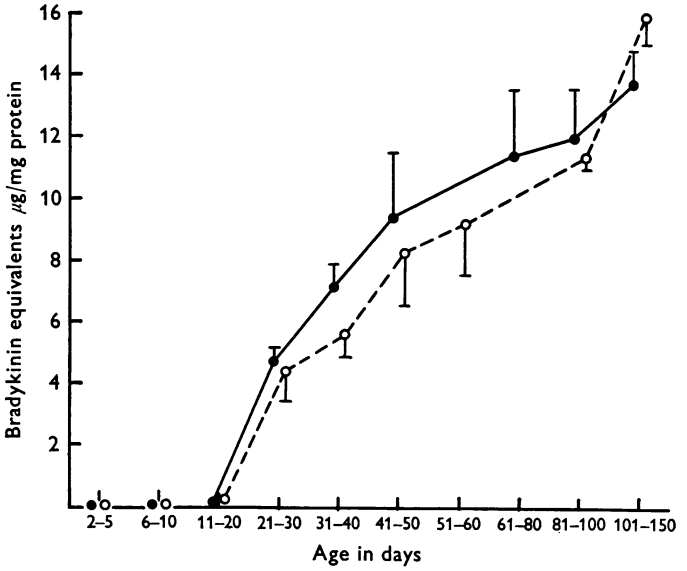
This difference was attributed to the increased activity of the androgen-influenced trypsin and to a lesser extent chymotrypsin in the extracts. However, unlike ATEe, CPPN and BAPA, the BAEe activity in the male continued to rise after 51–60 days post-natally. Part of this phase of the BAEe activity profile may have reflected kallikrein activity in the gland which increased with age up to 150 days post-natally (Text-fig. 5).



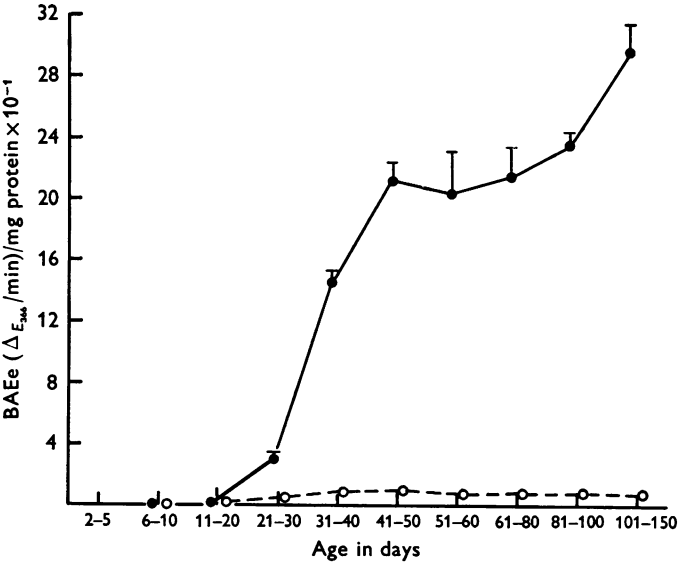
Text-fig. 2. Age- and sex-related differences in the activity of the trypsin-like enzyme. Values for male (●——●) and female (○- - -○) are expressed as specific activity and represent mean measurements (n = 3–9) of separate extracts of pooled, freeze-dried submaxillary glands. Bar shows 1 S.E. of mean.



Text-fig. 3. Age- and sex-related differences in the activity of renin. Values for male (●——●) and female (○- - -○) are expressed as specific activity and represent mean measurements (n = 5–19) of separate extracts of pooled, freeze-dried submaxillary glands. Bar shows 1 S.E. of mean.



Text-fig. 4. Age- and sex-related differences in the activity of kallikrein values for male (●——●) and female (○- - -○) are expressed as specific activity and represent mean measurements (n = 3–13) of separate extracts of pooled, freeze-dried submaxillary glands. Bar shows 1 S.E. of mean.



Text-fig. 5. Age- and sex-related differences in BAEe hydrolysing activity. BAEe provided an estimate of the total estero-protease activity. Values for male (●——●) and female (○- - -○) are expressed as specific activity and represent mean measurements (n = 3–9) of separate extracts of pooled, freeze-dried submaxillary glands. Bar shows 1 S.E. of mean.

Effect of castration in the male mouse

Enzyme activity was determined in submaxillary glands of immature mice (30–31 days old) castrated for 30, 65, 75 and 157 days. The chymotrypsin, trypsin and renin activities were almost abolished within 30 days of castration (Table 2). The androgen sensitive component of BAEe was similarly reduced. In addition, mice castrated after reaching maturity (59–64 and 86–89 days old) also showed a clear loss of these hormone-dependent activities.

In contrast, kallikrein activity showed no clear reduction in the immature mice castrated for 75 days (Table 2). In fact, the BAEe activity remaining after 75 days of castration was comparable to that observed in the female of similar age and probably represented the esterase activity attributable to kallikrein. Even in mature mice castrated for 30 days the kallikrein activity was not significantly affected.

Effect of testosterone in castrated male mice

Males (29–32 days old) were castrated and 30 days later injected subcutaneously with testosterone oenanthate (0·5 mg) three times at intervals of four days and testosterone propionate (0·1 mg) daily for five days. Both preparations effectively increased the activity of the chymotrypsin, trypsin and renin enzymes. The kallikrein activity was relatively unaffected (Table 3).

Experiments were also carried out to examine whether the increase in the androgen sensitive enzymes could be affected by cyproterone acetate, which inhibits testosterone action on target cells, or by oestradiol valerate. Immature male mice castrated for 30 days were injected subcutaneously with testosterone propionate (0·1 mg) alone and in combination with cyproterone acetate (3 mg) or oestradiol valerate (1 mg) daily for 5 days. The increase in the androgen-sensitive chymotrypsin and trypsin was unaffected by cyproterone acetate and by oestradiol valerate (Table 3).

Effect of testosterone in female mice

Immature female mice (30–31 days old) were injected subcutaneously with 10 mg testosterone oenanthate at 7-day intervals. The enzymes were estimated three days after the first dose and subsequently at varying periods. Chymotrypsin activity increased about 8000-fold in 55 days (Table 4). A similar, but very much less dramatic, increase was found with renin and trypsin. The increase in renin activity, however, began falling after 21 days; a similar reduction in chymotrypsin and trypsin was also found, but only after 67 days. In contrast, kallikrein activity decreased progressively in these testosterone-treated females (Table 4).

TABLE 2. Effect of castration on chymotrypsin, trypsin, renin and kallikrein activity in male mice

	Age at estimation (days)	Total esterases BAEe	Chymotrypsin ATEe	Trypsin BAPA	Renin	Kallikrein
A Control values	61–80	213 ± 17·9 (4)	7·49 ± 0·5 (6)	0·138 ± 0·012 (7)	4·8 ± 0·4 (10)	11·47 ± 2·1 (4)
	101–150	294·9 ± 17·6 (6)	6·03 ± 0·8 (8)	0·116 ± 0·006 (9)	4·67 ± 0·1 (5)	13·67 ± 1·0 (13)
B Castration at age 30/31 days						
Duration of castration (days)						
30	60–61	34·4 ± 2·4 (3)	0·071 ± 0·008 (4)	0·031 ± 0·002 (4)	0·141 ± 0·008 (5)	16·58 ± 0·9 (4)
63	93–94	12·6 ± 0·7 (4)	0·030 ± 0·002 (5)	0·016 ± 0·001 (4)	0·047 ± 0·002 (5)	n.t.
75	105–106	9·8 ± 1·0 (8)	0·022 ± 0·005 (4)	0·009 ± 0·006 (4)	0·040 ± 0·001 (5)	9·3 ± 0·43 (3)
157	187–188	2·5 ± 0·1 (4)	0·009 ± 0·001 (3)	n.t.	0·018 ± 0·001 (5)	n.t.
C Castration at age 59/64 days						
Duration of castration (days)						
30	99–104	19·2 ± 1·2 (4)	n.t.	0·035 ± 0·006 (4)	0·085 ± 0·003 (5)	17·3 ± 1·2 (8)

Enzyme values are expressed as specific activity; BAEe, ($\Delta_{E_{366\,nm}}$/min)/mg protein; ATEe, ($\Delta_{E_{366\,nm}}$/min)/mg protein; BAPA, (Δ_E405 nm/min)/mg protein; renin, angiotensin II equivalents – (μg/min)/mg protein; kallikrein, bradykinin equivalents – μg/mg protein. Figures represent mean measurements of separate extracts of pooled, freeze-dried submaxillary glands. ± S.E. of mean. n.t. = not tested.

TABLE 3. Effect of testosterone on enzyme activity in castrated male mice

	Age at estimation (days)	Chymotrypsin ATEe	Trypsin BAPA	Kallikrein
A Control values	31–40	5·83 ± 0·3 (10)	0·110 ± 0·003 (4)	7·19 ± 0·7 (3)
	61–80	7·49 ± 0·5 (6)	0·138 ± 0·012 (7)	11·47 ± 2·1 (3)
B Castration, values for 29–32 day-old mice castrated for 30 days	59–62	0·071 ± 0·008 (4)	0·031 ± 0·002 (4)	16·58 ± 0·9 (4)
C Castrated mice from *B* injected with				
(i) testosterone oenanthate	71–74	6·68 ± 0·64 (3)	0·093 ± 0·002 (4)	11·61 ± 1·5 (3)
(ii) testosterone oenanthate and oestradiol valerate	71–74	5·62 ± 0·68 (3)	0·101 ± 0·001 (3)	12·74 ± 2·1 (3)
(iii) testosterone propionate	64–67	2·76 ± 0·35 (3)	0·065 ± 0·005 (4)	10·43 ± 1·1 (3)
(iv) testosterone propionate and cyproterone acetate	64–67	2·54 ± 0·30 (4)	0·058 ± 0·003 (4)	13·56 ± 1·5 (3)

Enzyme values are expressed as specific activity: ATEe, ($\Delta_{E_{366\,nm}}$/min)/mg protein; BAPA, ($\Delta_{E_{405\,nm}}$/min)/mg protein; kallikrein, bradykinin equivalents – μg/mg protein. Figures represent mean measurements of separate extracts of pooled, freeze-dried submaxillary glands. ± S.E. of mean.

TABLE 4. Effect of testosterone oenanthate on enzyme activity in female mice

	Age at estimation (days)	Total esterases BAEe	Chymotrypsin ATEe	Trypsin BAPA	Renin	Kallikrein
A Control values	31–40	8·38 ± 0·6 (3)	0·003 ± 0·0005 (5)	0·008 ± 0·0002 (4)	0·061 ± 0·004 (5)	5·57 ± 0·7 (3)
	51–60	6·79 ± 0·6 (3)	0·006 ± 0·0007 (4)	0·005 ± 0·0003 (4)	0·093 ± 0·003 (5)	9·24 ± 1·7 (3)
	101–150	6·08 ± 0·5 (3)	0·001 ± 0·0003 (7)	0·005 ± 0·0004 (5)	0·048 ± 0·003 (5)	15·75 ± 0·9 (12)
B Female mice injected with testosterone oenanthate Duration in days						
3	41–42	34·5 ± 5·9 (4)	1·36 ± 0·1 (4)	0·053 ± 0·001 (4)	2·16 ± 0·043 (5)	n.t.
10	48–49	94·5 ± 2·2 (5)	8·61 ± 1·2 (3)	0·120 ± 0·003 (4)	3·44 ± 0·06 (5)	n.t.
21	59–60	214·3 ± 33·3 (3)	14·65 ± 2·2 (3)	0·162 ± 0·004 (4)	5·86 ± 0·15 (5)	8·02 ± 1·5 (4)
55	83–84	231·9 ± 33·7 (3)	33·22 ± 6·0 (3)	0·211 ± 0·018 (3)	3·25 ± 0·16 (5)	1·86 ± 0·7 (3)
67	105–106	328·2 ± 37·7 (5)	22·87 ± 0·7 (3)	0·203 ± 0·006 (4)	2·89 ± 0·10 (5)	0·88 ± 0·06 (3)

Enzyme values expressed as specific activity; BAEe, ($\Delta_{E_{366\,nm}}$/min)/mg protein; ATEe, ($\Delta_{E_{366\,nm}}$/min)/mg protein; BAPA, ($\Delta_{E_{405\,nm}}$/min)/mg protein; renin, angiotensin II equivalents – (μg/min)/mg protein; kallikrein, bradykinin equivalents – μg/mg protein. Figures represent mean measurements of separate extracts of pooled, freeze-dried submaxillary glands. ± S.E. of mean. n.t. = not tested.

Morphology

Age- and sex-related differences

Submaxillary glands in the adult male mouse are characteristically larger than in the female and contain a larger proportion of granular secretory tubules. The secretory granules in the acinar cells of the male and female are very similar, unlike the granules in the intercalated duct and secretory tubule which show sex-related differences (Fig. 6*a* and *b*; Table 5). In the male, granules are absent from cells of the intercalated duct (Pl. 1 A), whereas in the female the cells in the proximal section of the duct contain many granules of varying size and density (Pl. 1 *B*).

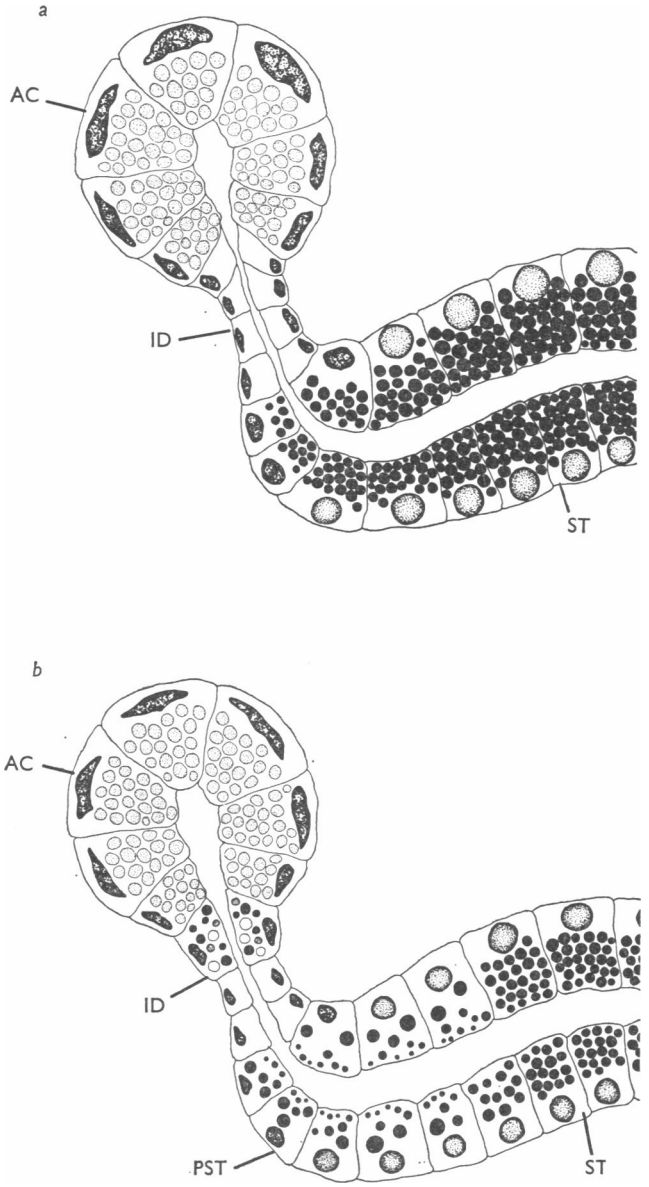
TABLE 5. Comparison of granule size in experimental and control male and female mouse submaxillary glands

	Acinus		Intercalated duct		Striated tubule		Secretory tubule	
	M	F	M	F	M	F	M	F
Control	2·2	2·3	*	1·4	*	1·5	2·6	2·2
After male castration								
1 month	—	—	—	—	—	—	1·4	—
2 months	No change		—	—	—	—	1·4	—
4 months	—	—	1·2	—	—	—	1·1	—
After testosterone administration for 1 month in the female	No change		—	*	—	*	—	2·7

M = male, F = female, * denotes absence of granules. Measurements are of mean diameters in μm.

The secretory tubule in the male has a uniform structure consisting predominantly of one type of cell in which very large numbers of dense granules are present (Pl. 2 *A*). In the female, however, the secretory tubule can be divided into two regions, a proximal portion in which the cells contain only a few, mainly dense granules and show characteristic basal striations (Pl. 2 *B*), and a shorter, distal portion in which the cells contain a larger number of dense granules but often lack striations (Caramia, 1966*a*).

For the first 20 post-natal days there is no apparent structural difference between male and female glands. At birth the secretory part of the gland consists of foetal terminal tubule cells which contain a large number of polymorphic, secretory granules. Acinar cells develop from terminal tubule cells within the first few days. They contain pale granules which for a short time possess a characteristic acentric dense core. Secretory tubule cells are devoid of granules until about 15 days after birth when



Text-fig. 6. Morphological comparison between *a* male and *b* female mouse submaxillary glands. AC = acinus; ID = intercalated duct; ST = secretory tubule; PST = proximal striated tubule (in female only).

a small number begin to appear. The cells in both sexes show a marked basal striation and a centrally placed nucleus. At this stage acinar tissue takes up the larger part of the gland.

After 20 days the structure of the gland begins to show sex-related differences. In the female, the gradual and uniform development of the acinar, intercalated and secretory tubule cells is maintained, but in the male the terminal tubule cells become reduced in number as the secretory tubules begin to occupy an increased area of the gland.

The main divergence occurs at 35–40 days of age when a series of rapid changes take place in the structure of the male gland. First, the secretory tubules of the male occupy a proportionately greater part of the gland. In the female acinar tissue remains more abundant. Second, a massive population of dense granules develops in the male tubule cells resulting in the nucleus being moved from a central position in the cell to one near the basal membrane. Third, the endoplasmic reticulum in the male tubule cells becomes characteristically vesicular. Fourth, the cells of the terminal tubules disappear in the male whereas in the female they persist, becoming the granule-containing cells of the intercalated duct.

Effect of castration in the male mouse

After castration the structure of both the secretory tubule and the intercalated duct cells is changed (Caramia, 1966*b*). One month after castration (30–31 day-old mice) the secretory tubules are smaller in diameter and the cells have lost the vesicular type of endoplasmic reticulum and contain fewer granules (see Table 2 for corresponding enzyme values). Four months after castration, granules are absent from many tubule cells and when present are considerably reduced in size (Pl. 3 *A*; see Pl. 2 *A* for comparison). The secretory tubule cells regain the basal striations lost in maturity. In addition, the normally granule-free cells of the intercalated ducts become filled with a granule population similar to that of the female intercalated ducts (Pl. 3 *B*; see Pl. 1 *B* for comparison). The structure of both the secretory tubule and the intercalated ducts reverts, therefore, to the condition found in the juvenile state. The acinar cells remain unchanged but because of the reduction in size of the secretory tubules they appear to occupy a greater part of the gland.

Effect of testosterone on castrated male mice

The glands of male mice (29–32 days old) castrated for 30 days and then injected with testosterone oenanthate or testosterone propionate show a substantial return to the normal male structure (see Table 3 for corresponding enzyme values). Secretory tubules are larger and the granule population increases to approximately the same numbers present in sham

operated controls. The injection of cyproterone acetate does not appear to prevent the return of the gland structure to that observed in the normal male.

Effect of testosterone on female mice

After 1 month of injecting testosterone oenanthate into 38–39 days old female mice the appearance of the gland is considerably changed (see Table 4 for corresponding enzyme values). The normal structure of the proximal and distal secretory tubules is lost and the distinctions between them are completely obliterated. The cells become filled with huge dense granules, many of which are larger than those seen in the normal male gland (Pl. 4 *A*; for comparison see Pls. 2 *B* and 2 *A*). The nucleus is forced into a basal position and the endoplasmic reticulum becomes vesicular in type. In addition, the granules of the intercalated duct cells disappear (Pl. 4 *B*; for comparison see Pl. 3 *B*). The pale granule population of the acinar cells remains unaffected but because of the immense proliferation of the secretory tubule cells in both size and quantity, the area taken up by the acinar tissue is considerably reduced.

DISCUSSION

The serine proteases chymotrypsin, trypsin and kallikrein effectively hydrolyse synthetic esters of various amino acids. Their relative ability to cleave particular synthetic substrates was therefore used to identify and characterize these proteases in extracts of mouse submaxillary gland. The chymotrypsin-like enzyme was identified by its esterase activity on ATEe and amide activity on the more specific, but somewhat insensitive, substrate CPPN. It was considered unlikely that the esterase activity of submaxillary kallikrein and trypsin which act mainly on esters of arginine could have accounted for a significant proportion of the tyrosine ester (ATEe) activity in extracts of the male gland. A renin contribution was also excluded because a partially purified, hog kidney renin preparation (Miles Serevac) failed to hydrolyse ATEe, CPPN, BAPA or BAEe. The specific amide activity of the gland extracts on BAPA was used to confirm the presence of a trypsin-like enzyme. The kallikrein and renin activities were determined by clearly established bio-assay methods. Experiments are in progress to isolate, purify and chemically characterize further these enzymes.

The trypsin-like enzymes isolated from the submaxillary gland of the mouse by Angeletti, Angeletti & Calissano (1967) and the rat by Riekkinen *et al.* (1966, 1967) are inhibited by aprotinin (see also Bhoola & Dorey, 1971*a*). In this study whereas the activity of the trypsin-like protease (on BAPA) was similarly blocked by aprotinin (trasylol), the activity of

the chymotrypsin-like enzyme (on ATEe, aprotinin 3300 u./ml.) was relatively unaffected (unpublished results).

The relative esterase activity, the specificity on amide substrates and the inhibition profile with aprotinin provide sufficient evidence for the presence of distinct chymotrypsin- and trypsin-like proteases in the mouse submaxillary gland. The occurrence of a chymotrypsin-like enzyme has not previously been reported in mammalian submaxillary glands. The finding of this enzyme further focuses attention on the presence in mammalian submaxillary glands of proteases similar to those formed and secreted by the pancreas. The physiological importance of these enzymes remains uncertain.

The present study has clearly delineated the influence of age and androgens on the synthesis of individual enzymes, in particular the estero-proteases. In addition, the changes in enzyme levels have been correlated with the change in number and size of secretory granules in the various morphological units within the mouse submaxillary gland. Each of the five enzymes showed low activity at birth in both sexes. Kallikrein was the first enzyme to show an increase in activity, rising rapidly post-natally. We confirmed the finding of Werle (1960) that mouse submaxillary kallikrein is virtually absent at birth but begins to appear at the end of the first week of life. Recently, a similar post-natal change in submaxillary kallikrein has been demonstrated in the rat (Beraldo, Araujo, Siqueira, Rodrigues & Machado, 1971). The increase in kallikrein activity in the mouse coincided with the appearance of organized acinar structure in both the male and female gland. However, the consistent finding of a marginally higher kallikrein activity in the male (Fig. 5) might be explained if the trypsin-like protease possessed kininogenase activity. Although it is possible that the submaxillary gland trypsins may show some such activity, proof of it must await their purification. In contrast, the chymotrypsin, trypsin and renin activities increased rapidly only in the male and at a later age after birth. The biochemical sexual dimorphism which became manifest at maturity was accompanied by a marked increase in size and number of granules in the secretory tubules. Testosterone-associated increase in the weight of seminal vesicles of male mice taken from the colony used in this study (R. Sharpe, personal communication) followed a similar time course.

The androgen dependence of these enzymes was directly tested in castrated males. The concentration of the chymotrypsin and trypsin proteases and renin in the gland fell rapidly and this was accompanied by the disappearance of granules in the secretory tubules. On the other hand, the gland content of kallikrein and the organelles in the acinar tissue were unaffected by castration. Replacement of testosterone in the castrated

male resulted in a parallel return of the renin, chymotrypsin and trypsin activities. Treatment of the female with testosterone was accompanied by rapid changes in the three enzymes and in the intracellular structure. With continued administration of testosterone, as the marked proliferation of the secretory tubules progressed, the acinar tissue shrank and concomitantly the kallikrein activity fell, reaching, after two months of treatment, values which were 10% of those detected in control female glands.

The results clearly indicate that the synthesis of the chymotrypsin enzyme is regulated by testosterone in the same way as are renin and the trypsin proteases. The synthesis of the closely related estero-protease, kallikrein, is unaffected. This combined study provides strong evidence that kallikrein is synthesized in the acinar and perhaps also intercalated duct cells, whilst renin and the chymotrypsin and trypsin proteases are formed in the cells of the secretory tubules. Our results do not support the finding of Chang *et al.* (1968) that kallikrein and renin are stored in the same organelles, but are in agreement with previous subcellular studies which indicate that kallikrein is sequested in separate granules formed in the acinar cells (Bhoola & Heap, 1970; Bhoola & Dorey, 1971*b*).

Enzyme induction is believed to be regulated by specific genes which cycle between inducible and non-inducible phases. During the inducible phase the structural gene for the enzyme is transcribed and the resulting messenger translated to form the enzyme. At the same time the regulatory gene is also transcribed and its messenger translated to produce a specific labile repressor (Tomkins, 1971). Testosterone and steroid hormones in general are believed to regulate enzyme induction by inactivating the specific labile repressor. It was therefore of particular interest to examine whether cyproterone acetate, which is said to compete with the binding of dehydrotestosterone, a metabolite of testosterone, to receptors in the cytosol and the nuclear chromatin material (Fang, Anderson & Liao, 1969), would antagonize the induction and synthesis of the chymotrypsin and trypsin proteases. Cyproterone acetate not only failed to affect the increased formation of these enzymes, but also had no effect on the growth and proliferation of the secretory tubules. The question of why kallikrein, which belongs to the same family of estero-proteases as the chymotrypsin and trypsin enzymes, was not induced by testosterone remains unanswered.

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EXPLANATION OF PLATES

PLATE 1

A, male mouse submaxillary gland. Acinar cells (AC) contain pale granules. Intercalated duct cells (ID) are devoid of granules.

B, female mouse submaxillary gland. Acinar cells (AC) contain pale granules similar to those in the male. In comparison, however, cells of the intercalated duct (ID) adjacent to the acinus contain many granules of varying density.

PLATE 2

A, male mouse submaxillary gland. Secretory tubule cells (ST) contain many large dense granules and a characteristically vesicular endoplasmic reticulum.

B, female mouse submaxillary gland. Proximal striated tubule cells (PST) contain a few apically placed granules and show characteristic basal striations (arrow).

PLATE 3

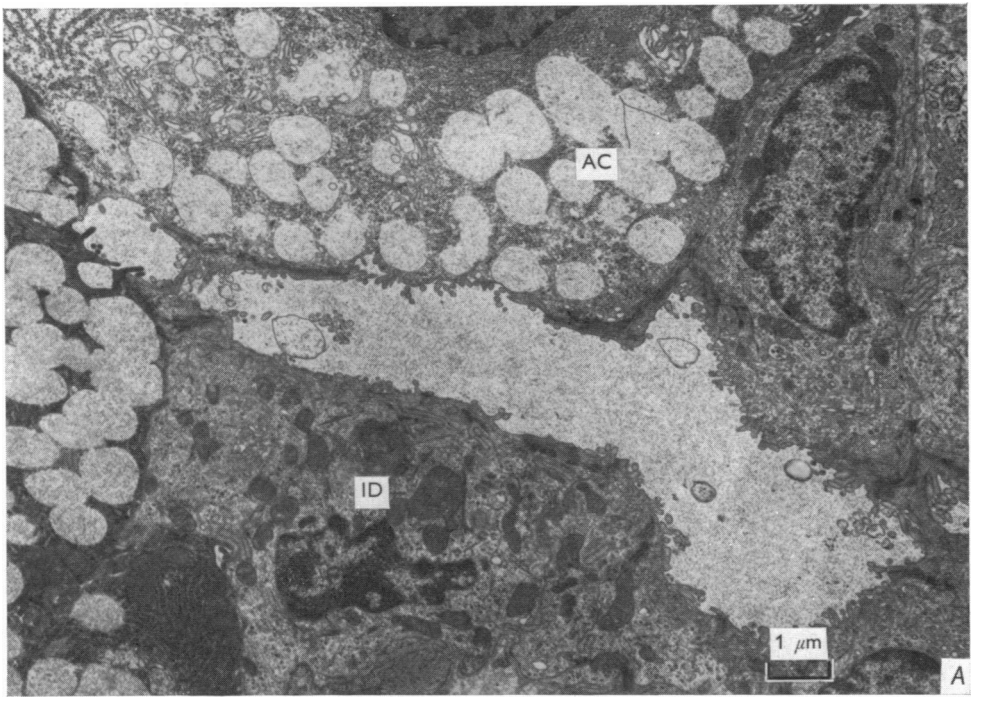
A, male mouse submaxillary gland, 4 months after castration. Granules of the secretory tubule cells (ST) are considerably reduced in size and may be absent from some cells. Basal striations have appeared (arrow).

B, male mouse submaxillary gland, 4 months after castration. Granules are now present in the intercalated duct cells (ID). Acinar cell granules (AC) have not changed.

PLATE 4

A, female mouse submaxillary gland, 1 month after testosterone administration. The characteristics of the proximal striated tubule cells have disappeared. Instead the cell structure is similar to the male secretory tubule, with large dense granules and vesicular endoplasmic reticulum (arrow).

B, female mouse submaxillary gland, 1 month after testosterone administration. Cells of the intercalated duct (ID) are now devoid of granules. Acinar cell granules (AC) have not changed.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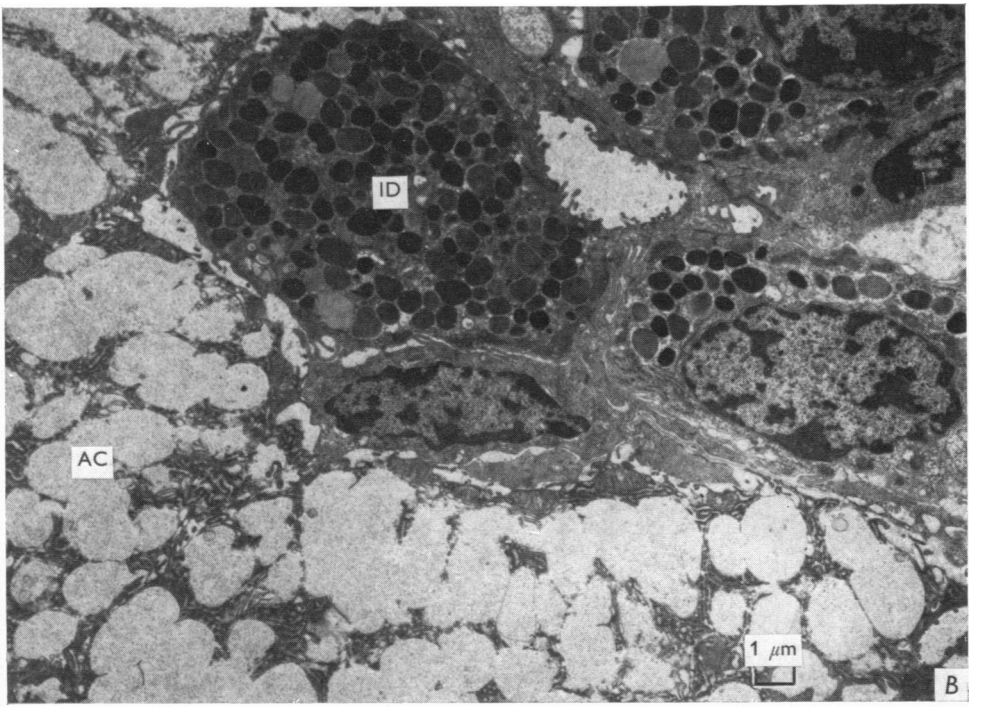
A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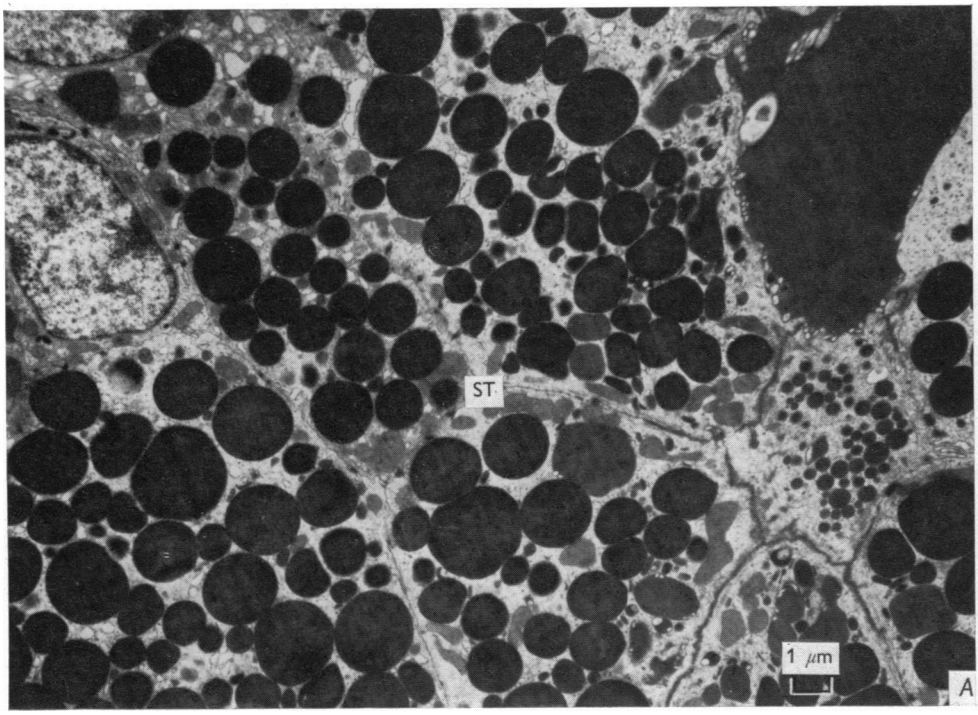
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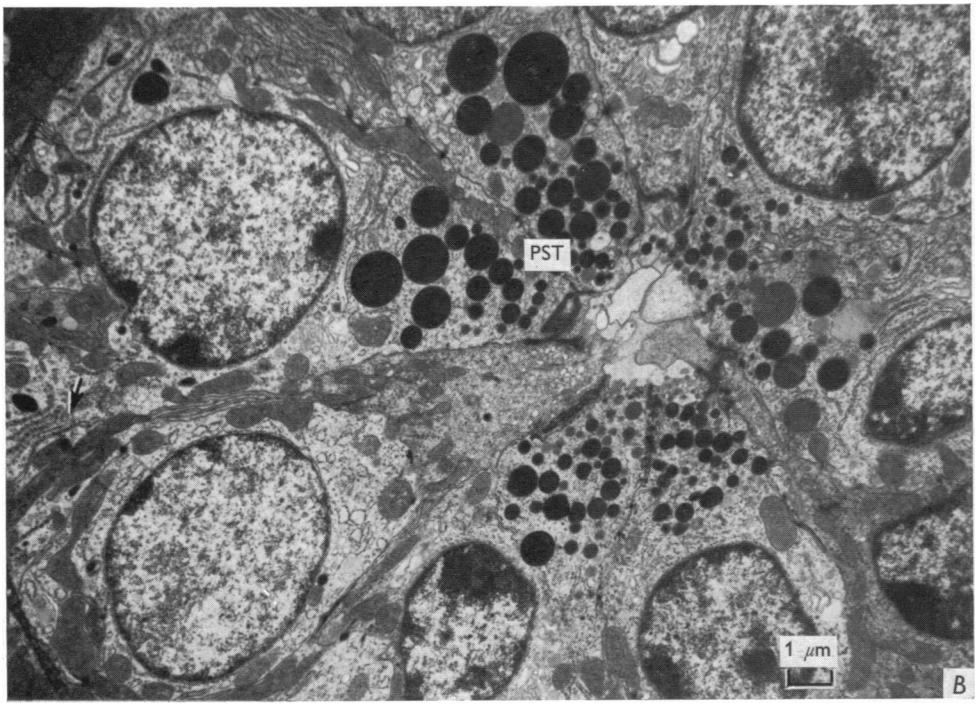
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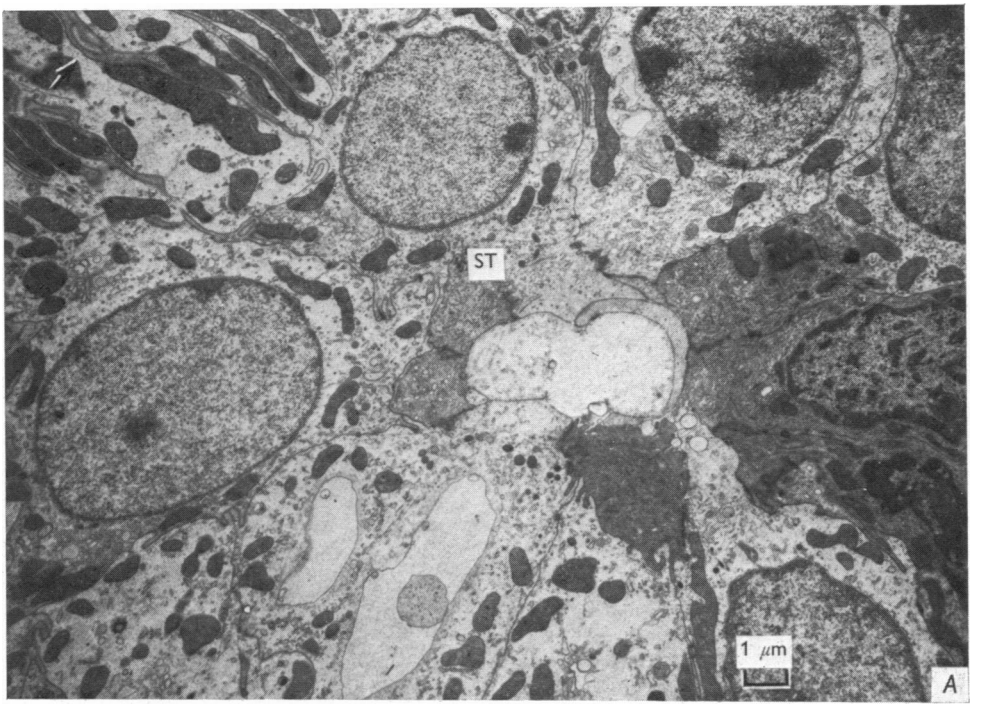
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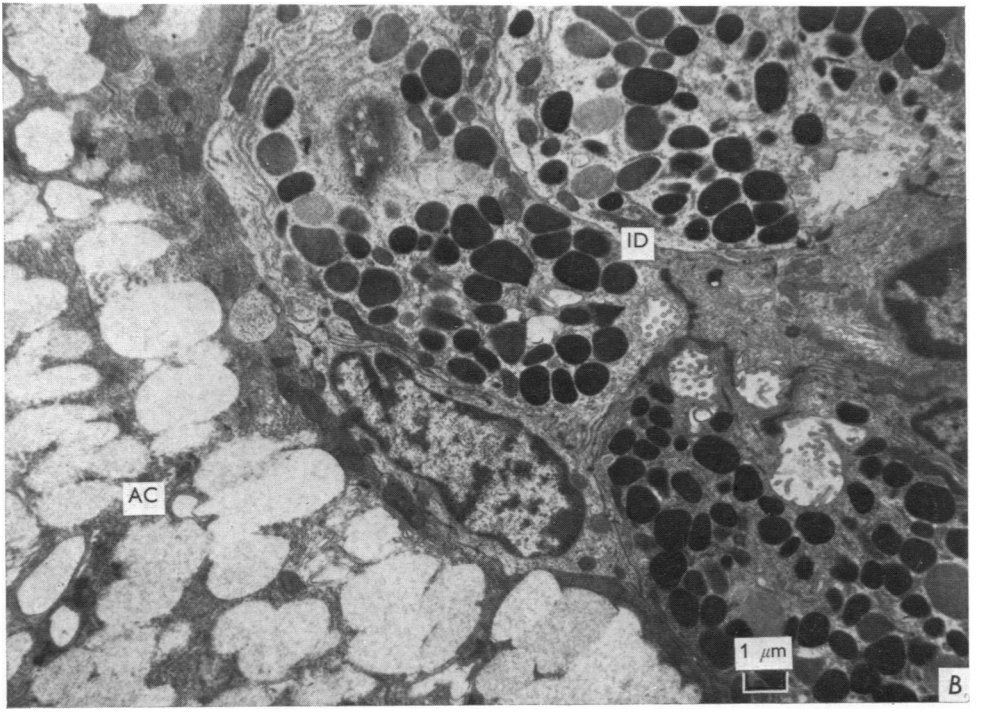
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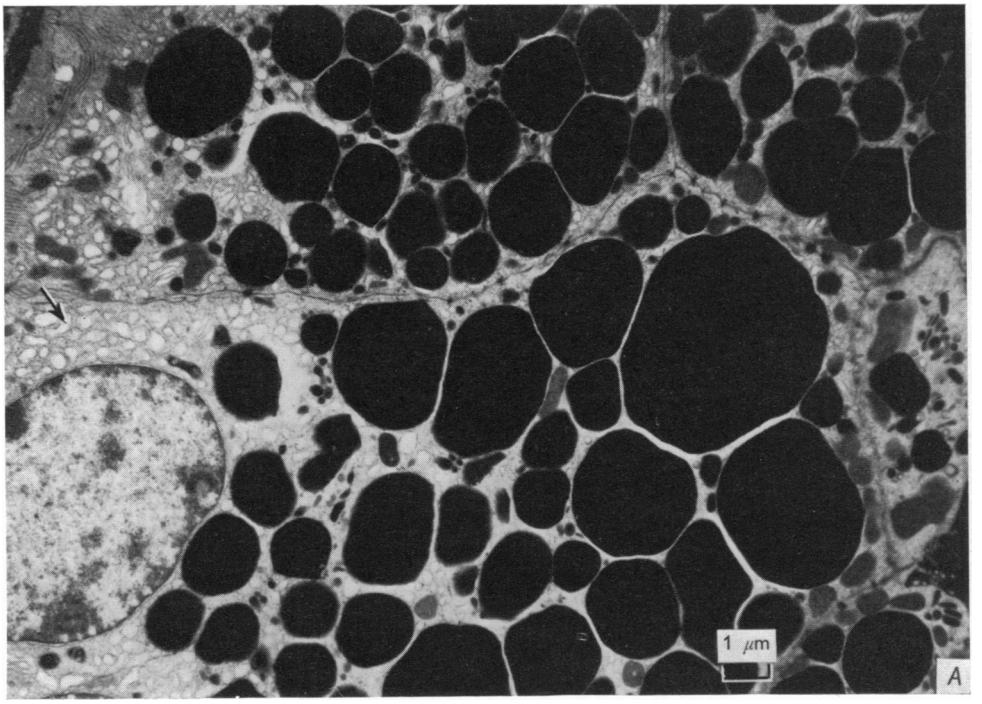
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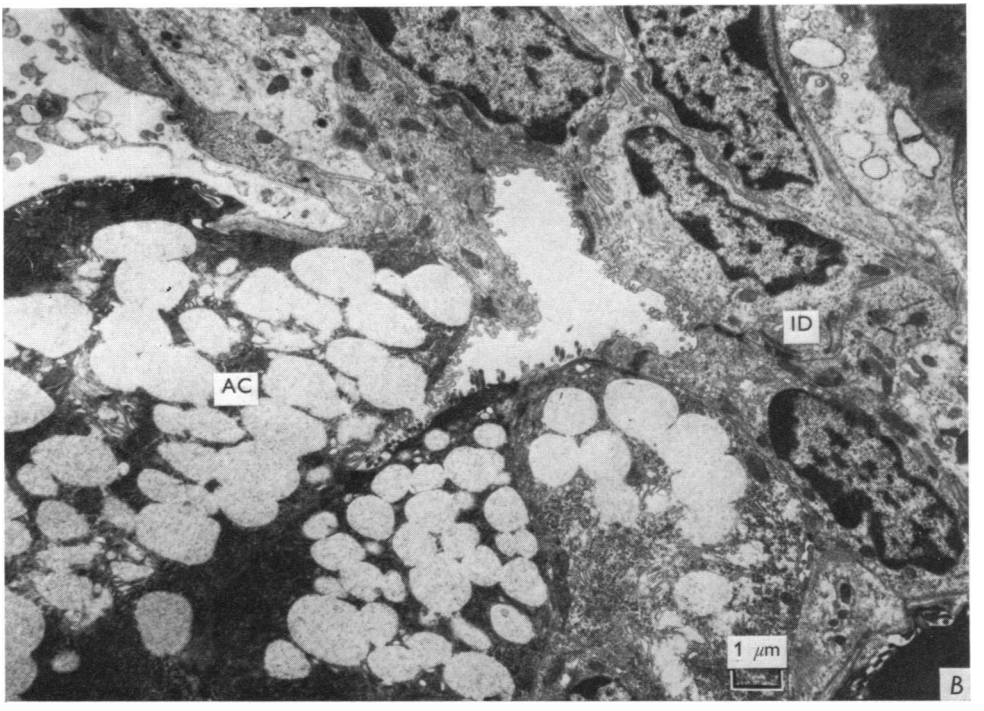
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