J. Physiol. (1968), **196**, *pp.* 431–445
With 1 *plate and* 3 *text-figures*
Printed in Great Britain

INTRACELLULAR DISTRIBUTION OF SUBMAXILLARY KALLIKREIN

BY K. D. BHOOLA*

From the Department of Pharmacology, Middlesex Hospital Medical School, London, W. 1

(*Received* 30 *November* 1967)

SUMMARY

1. Differential centrifugation and sucrose density-gradient data indicate that kallikrein in the submaxillary gland is associated with particles which sediment with the nuclear and heavy mitochondrial fractions and equilibrate with 1·7–1·8 M sucrose.

2. The sedimentation properties of the kallikrein containing particles differ from those of mitochondria and lysosomes, but resemble those of the pancreatic zymogen granules.

3. Histological observations indicate that kallikrein is associated with basophilic particles which stain positively with Periodic Acid-Schiff reagent.

4. Pilocarpine causes a preferential secretion of kallikrein and amylase into saliva.

5. The results would support an exocrine role for submaxillary kallikrein.

INTRODUCTION

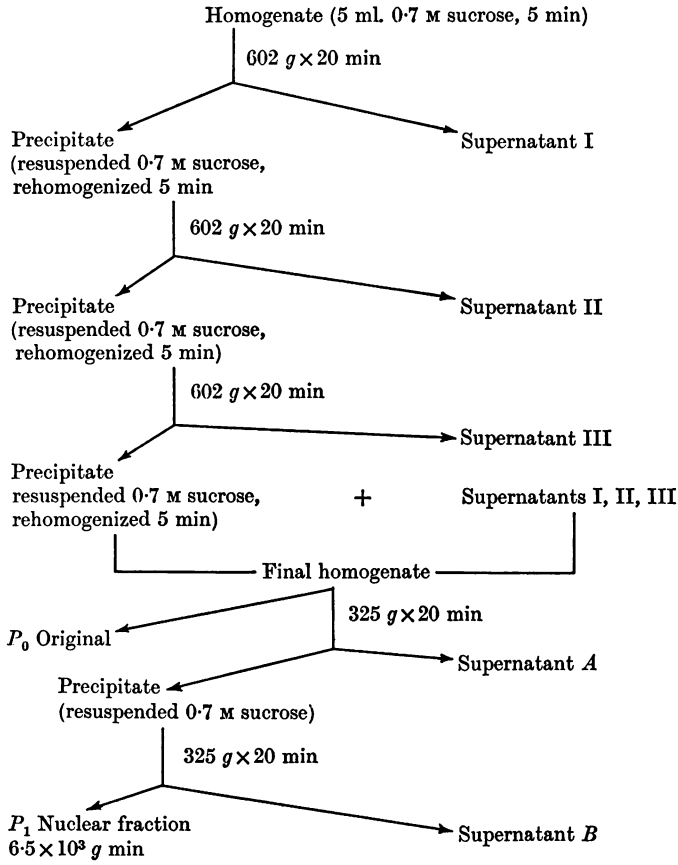
On differential centrifugation kallikrein was localized in the nuclear and mitochondrial fractions of submaxillary gland homogenates (Bhoola & Ogle, 1966) and its sedimentation properties were similar to those of pancreatic zymogen granules (Siekevitz & Palade, 1958*a*). In the present experiments the previous observations on the subcellular distribution of submaxillary kallikrein have been extended and compared in greater detail with reference to mitochondrial and lysosomal enzymes. These experiments provide a useful approach in determining the physiological function of the glandular kallikreins.

* Present address: Department of Pharmacology, University of Bristol, Bristol 8.

METHODS

Preparation of homogenates

Submaxillary glands from four to six guinea-pigs were dissected, rinsed in ice-cold 0·7 M sucrose, dried on filter paper and weighed. The glands were cut into small pieces and homogenized with a M.S.E. homogenizer (Text-fig. 1). The concentration of fresh gland tissue in the homogenate ranged from 27 to 38 mg/ml.



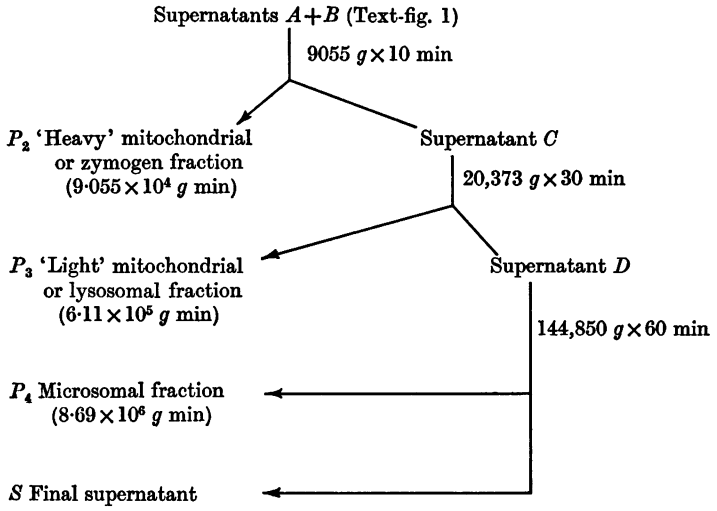
Text-fig. 1. Differential centrifugation (M.S.E.)

Differential centrifugation

The nuclear fraction was separated on a M.S.E. centrifuge using rotor no. 6886 (Text-fig. 1). Subsequent fractionation was carried out in the Spinco-Model L-2 preparative centrifuge with rotor no. 40 (Text-fig. 2). The fractionation scheme used was adapted from that previously described for pancreas (Siekevitz & Palade, 1958*a*) and liver (Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) (Table 1).

Density-gradient centrifugation

Density-gradient centrifugation was carried out on the heavy mitochondrial fraction P_2 (see Table 1) using the swinging-bucket Spinco rotor no. 39L. The fraction was suspended in 0·7 M sucrose and 1·5 ml. (mean: 32 mg protein/ml.) was layered on a 4 ml. column of 0·5 ml. or 1·0 ml. gradients. The tubes were centrifuged either at $1{\cdot}47 \times 10^6$ *g* min or at $10{\cdot}51 \times 10^6$ g min. For further separation of the enzymes, 1·5 ml. fraction P_2 (mean: 33 mg protein/ml.)



Text-fig. 2. Differential centrifugation (Spinco-Model L-2).

TABLE 1. Comparison of gravitation forces

Fractions	Tissues: Sub-maxillary gland	Pancreas*	Liver†	Gravitational force
P_1 Nuclear	6·5	7·6	6·0	$\times 10^3$ g min
P_2 Heavy mitochondrial zymogen	9·0	8·6	3·3	$\times 10^4$ g min
P_3 Light mitochondrial lysosomal	6·1	6·0	2·5	$\times 10^5$ g min
P_4 Microsomal	8·6	6·3	3·0	$\times 10^6$ g min
Sucrose molarity	0·7	0·88	0·25	—

* Siekevitz & Palade (1958*a*). † Duve *et al.* (1955).

was layered on 4 ml. of 1·6 M sucrose and centrifuged at $1{\cdot}47 \times 10^6$ *g* min; the precipitate was suspended in 1·6 M sucrose and 1·5 ml. (6·1 mg protein/ml.) was layered on a 4 ml. column of sucrose gradients and centrifuged at $10{\cdot}51 \times 10^6$ *g* min.

The discontinuous sucrose gradients were prepared 1–2 hr before use and allowed to equilibrate at 4° C. The junction of each layer was marked and after centrifugation the tubes were cut with a specially designed cutter which permitted separation and collection of each sucrose layer.

Histological observation

Fraction P_2 was centrifuged on a column of 1·6 M sucrose at $1{\cdot}47 \times 10^6$ *g* min (see density-gradient centrifugation). Smears of the precipitate (P_2p) were stained with Janus Green and

examined after 10 min. Smears were also made on albumenized slides and fixed with 10% neutral formal saline. They were stained with haematoxylin and eosin, Periodic Acid-Schiff (P.A.S.) reagent and Oil Red O.

Unhomogenized guinea-pig submaxillary gland was also examined histologically; the tissue was fixed in Bouin's fluid, embedded in wax, sectioned and stained for light microscopy.

Stimulation by pilocarpine

Guinea-pigs, which had been allowed food *ad libitum*, were anaesthetized with sodium pentobarbitone (20 mg/kg), injected intraperitoneally. The animals were placed in recumbent posture with the head in dependent position. The mouth was thoroughly rinsed with saline (0·9 g/100 ml.). Pilocarpine nitrate (10 mg/kg) was injected subcutaneously at 15 min intervals for 1 hr. Saliva was collected with a Pasteur pipette, kept at 4° C, and later centrifuged; the supernatant was assayed for the intracellular enzymes estimated in this study.

After the collection of saliva was completed, the animals were killed by a blow on the head and the submaxillary glands removed for subcellular fractionation. The homogenization and fractionation procedure was the same as that used for the non-stimulated glands. The concentration of fresh gland tissue ranged from 28 to 37 mg/ml.

Assay of enzymes

Kallikrein activity was measured by its ability to release kallidin from heated (61° C, 3 hr), dialysed dog or human plasma; both kininase and the precursor of kallikrein are destroyed in plasma prepared in this way (Holdstock, Mathias & Schachter, 1957). The activity of the released kallidin was measured on the isolated guinea-pig ileum preparation. Samples were match-assayed against the original homogenate or appropriate sub-fraction in the case of the sucrose density-gradient fractions. The activity of each fraction was expressed as a percentage of the activity in the original homogenate or subfraction.

The guinea-pig ileum preparation was suspended in Tyrode solution (mM: NaCl, 154; $MgCl_2$, 2·1; $CaCl_2$, 1·8; $NaHCO_3$, 11·9; glucose, 5·56) at 35° C and bubbled with 95% O_2–5% CO_2 mixture. Mepyramine (10^{-8} g/ml.) and atropine (10^{-8} g/ml.) were added to the bath fluid before each test. Because of the kininase content of the various subcellular fractions (K. D. Bhoola & C. W. Ogle, unpublished), kallikrein activity was also measured in the presence of the kininase inhibitor (−)-cysteine ($2·5–5 \times 10^{-5}$ g/ml.). The assays with and without (−)-cysteine gave similar distribution curves for kallikrein.

Succinic dehydrogenase was measured manometrically by the micro-spirometer method (Whittaker, 1959; cf. Bhoola & Ogle, 1966). The amount of CO_2 produced in μl./hr was used to calculate activity.

β-Glucuronidase was estimated by the method of Kerr & Levy (1951). A 1 ml. fraction was added to 1 ml. 0·3 M acetate buffer (pH 5·0) and 1 ml. phenolphthalein β-D-glucuronide (0·005 M in acetate buffer). After incubation at 37° C for 1 hr the reaction was terminated by adding 6 ml. alkaline reagent (pH 10·7; 1 M-Na_2CO_3, 0·4 M glycine and 0·2 M-NaCl). The mixture was centrifuged and read at 550 mμ on the Hilger spectrophotometer.

Acid phosphatase was measured initially by the method of Berthet & Duve (1952) using sodium β-glycerophosphate as substrate. Because this method proved relatively insensitive for submaxillary tissue, modification of the procedure described by Laurent & Norberg (1960) was adopted even though glucose-6-phosphatase activity was not excluded. A fraction of 1 ml. was added to 1 ml. 0·2 M citrate buffer (pH 4·9), 0·1 ml. 1% Tritton × 100 and 1 ml. disodium *p*-nitro phenyl orthophosphate (0·001 M or 0·005 M in citrate buffer). The mixture was incubated at 37° C and after 1 hr the reaction stopped with 1 ml. 8% trichloracetic acid. Next 2 ml. of 15% Na_2CO_3 was added and the tubes were incubated at 37° C for 10 min for full development of colour. The mixture was centrifuged and read at 420 mμ on the Hilger spectrophotometer.

Ribonuclease and uricase were estimated by the method of Duve *et al.* (1955).

Amylase was measured by the iodine titration method. The incubation mixture was the same as that described previously (Bhoola & Ogle, 1966). This mixture (0·1 ml.) was added every 1 min to 3 ml. of 0·02 N iodine solution and read on a Linson 3 photometer (filter no. 610). The time taken to reach an optical density change from 1·0 to 0·3 (50 % transmission) was used to calculate amylase activity.

Protein estimation

Protein was measured by the method of Lowry, Roseburgh, Farr & Randall (1951).

Expression of activities

The activity of each enzyme and of protein was expressed as a percentage of total recovery (PTR). The relative specific activity (RSA) was calculated by dividing the percentage activity (PTR) by the protein content of the same fraction expressed as a percentage of the total recovered protein.

The protein and enzyme activity in saliva were expressed as percentages of the activity in the submaxillary gland homogenate (PO); and specific activity (SA) was determined by dividing the percentage enzyme activity by the percentage protein content.

RESULTS

Comparison of the subcellular distribution of kallikrein with that of other intracellular enzymes

Succinic dehydrogenase. This enzyme reflects the distribution of mitochondria and serves as a useful reference enzyme for subcellular studies (Whittaker, 1965). In the present experiments succinic dehydrogenase was mainly located in the heavy (40·0%) and light (27·7 %) mitochondrial fractions; these together corresponded to the value observed previously in the mitochondrial fraction of the submaxillary gland (Bhoola & Ogle, 1966). This distribution was similar to that described for cytochrome oxidase and succinate-(cytochrome c)-reductase in liver (Duve *et al.* 1955) (Table 2).

The distribution of kallikrein was different. Particulate kallikrein was mostly confined to the nuclear (32·7 %) and heavy mitochondrial (31·3 %) fractions. Its distribution pattern resembled that reported for the precursors of pancreatic trypsin and chymotrypsin, held in zymogen granules (Siekevitz & Palade, 1958*a*) (Table 3).

The microsomal fraction contained regularly a small amount of kallikrein (2·6 %). In experiments reported previously (Bhoola & Ogle, 1966) no kallikrein activity was detected in the microsomal fraction. However, the previous study unlike the present one was carried out on animals fasted for 12 hr before preparation of homogenates. A similar variation dependent on feeding has been described for pancreatic digestive enzymes (Siekevitz & Palade, 1958*b*).

Lysosomal acid hydrolysases and uricase. Of the lysosomal enzymes β-glucuronidase and acid phosphatase were estimated regularly. Table 4 shows their subcellular distribution. The very much greater recovery of these

TABLE 2. Comparison of the subcellular distribution of kallikrein with that of mitochondrial enzymes

Fraction	*g* min (0·70 M sucrose)	Kallikrein		Succinic dehydrogenase		*g* min (0·25 M sucrose)*	Cytochrome oxidase PTR*	Succinate-(cytochrome c)-reductase PTR*
		PTR	RSA	PTR	RSA			
P_1	$6{\cdot}5 \times 10^3$	$32{\cdot}7 \pm 5{\cdot}5$	3·37	$14{\cdot}8 \pm 3{\cdot}4$	1·66	$6{\cdot}0 \times 10^3$	$10{\cdot}1 \times 5{\cdot}4$	$10{\cdot}1 \pm 6{\cdot}5$
P_2	$9{\cdot}0 \times 10^4$	$31{\cdot}3 \pm 2{\cdot}2$	2·75	$40{\cdot}0 \pm 3{\cdot}9$	3·50	$3{\cdot}3 \times 10^4$	$57{\cdot}7 \pm 12{\cdot}0$	$49{\cdot}1 \pm 19{\cdot}0$
P_3	$6{\cdot}1 \times 10^5$	$7{\cdot}6 \pm 1{\cdot}4$	0·75	$27{\cdot}7 \pm 4{\cdot}1$	3·53	$2{\cdot}5 \times 10^5$	$17{\cdot}4 \pm 8{\cdot}7$	$9{\cdot}9 \pm 7{\cdot}1$
P_4	$8{\cdot}6 \times 10^6$	$2{\cdot}6 \pm 1{\cdot}5$	0·14	$11{\cdot}4 \pm 2{\cdot}7$	0·62	$3{\cdot}0 \times 10^6$	$3{\cdot}7 \pm 1{\cdot}6$	$17{\cdot}0 \pm 0{\cdot}4$
S	—	$25{\cdot}3 \pm 1{\cdot}2$	0·54	$5{\cdot}7 \pm 3{\cdot}0$	0·07	—	0	$1{\cdot}9 \pm 1{\cdot}9$
Total recovery (%)		99·2	—	73·4	—	—	88·9	72·7
No. of experiments		4	—	4	—	—	17	3

* Results of Duve *et al.* (1955) for liver tissue.

PTR: percentage total recovery. RSA: relative specific activity.

enzymes from the supernatant when compared with that of succinic dehydrogenase and with their localization in the liver (Duve *et al.* 1955) was probably due to greater lability of the lysosomal membrane to the homogenization technique used in these experiments. Several attempts to preserve the lysosomal membrane by using gentler techniques failed to produce satisfactory homogenates; clearly the lysosomes were more labile than mitochondria.

TABLE 3. Comparison of the subcellular distribution of kallikrein with that of the enzymes held in pancreatic zymogen granules

Fraction	*g* min (0·70 M) sucrose)	Kallikrein PTR	Kallikrein RSA	*g* min (0·88 M) sucrose)	TAPase PTR*	Chymo PTR*
P_1	$6·5 \times 10^3$	32·7	3·37	$7·6 \times 10^3$	28	26
P_2	$9·0 \times 10^4$	31·3	2·75	$8·6 \times 10^4$	59	36
P_3	$6·1 \times 10^5$	7·6	0·75	$6·0 \times 10^5$	4	18
P_4	$8·6 \times 10^6$	2·6	0·14	$6·3 \times 10^6$	6	12
S	—	25·3	0·54	—	3	8

* Results of Siekevitz & Palade (1958*a*) for pancreatic tissue.

PTR: percentage total recovery. RSA: relative specific activity.

TAPase: trypsin activated proteolytic activity. Chymo: chymotrypsinogen.

TABLE 4. Comparison of the subcellular distribution of kallikrein with that of lysosomal enzymes

Fraction	Kallikrein PTR	Kallikrein RSA	β-Glucuronidase PTR	β-Glucuronidase RSA	Acid phosphatase PTR	Acid phosphatase RSA
P_1	32·7 ± 5·5	3·37	9·8 ± 2·5	0·97	14·6 ± 1·8	1·59
P_2	31·3 ± 2·2	2·75	20·1 ± 3·2	1·97	17·9 ± 1·4	1·56
P_3	7·6 ± 1·4	0·75	23·3 ± 2·3	3·17	15·0 ± 1·5	1·53
P_4	2·6 ± 1·5	0·14	13·2 ± 6·0	0·64	16·3 ± 2·2	0·90
S	25·3 ± 1·2	0·54	33·3 ± 8·5	0·73	38·3 ± 5·6	0·75
Total recovery (%)	99·2	—	92·9	—	80·3	—
No. of experiments	4	—	4	—	4	—

PTR: percentage total recovery. RSA: relative specific activity.

In one experiment the localization of ribonuclease and of uricase was also followed and the results are shown in Table 5.

None of these enzymes gave distribution curves comparable to that of kallikrein.

Amylase. As shown previously (Bhoola & Ogle, 1966), the major amylase activity was recovered from the supernatant and only small amounts were located in the fractions (Table 6). The close correlation with previous observations suggests that amylase in the subcellular fractions may be held in particulate or bound form.

The localization of amylase in this study differed completely from that reported for pancreatic amylase (Hokin, 1955); the latter showed a distri-

bution pattern which closely resembled that observed for submaxillary kallikrein in the present experiments (Table 6).

Effect of pilocarpine on the intracellular enzymes

Pilocarpine produced little or no effect on the subcellular distribution of kallikrein and succinic dehydrogenase (Table 7). There was, however, a slight reduction in the activity of β-glucuronidase and acid phosphatase

TABLE 5. Subcellular distribution of ribonuclease and uricase

	Ribonuclease		Uricase	
Fraction	PTR	RSA	PTR	RSA
P_1	9·9	1·52	0	0
P_2	23·2	1·57	21·1	1·51
P_3	19·9	2·26	36·4	4·38
P_4	23·0	1·35	23·5	1·46
S	23·8	0·50	18·8	0·33
Total recovery (%)	144	—	118	—

PTR: percentage total recovery. RSA: relative specific activity.

TABLE 6. Comparison of the subcellular distribution of submaxillary amylase and kallikrein with that of pancreatic amylase

	Submaxillary tissue				Pancreatic tissue*	
Fraction	*g* min (0·70 M sucrose)	Amylase PTR	Kallikrein PTR	Fraction (0·25 M sucrose)	*g* min	Amylase PTR
P_1	$6{\cdot}5 \times 10^3$	1·9	32·7	P_1	6×10^3	35·6
P_2	$9{\cdot}0 \times 10^4$	3·4	31·3	P_2	2×10^4 $8{\cdot}5 \times 10^4$	23·3 13·3
P_3	$6{\cdot}1 \times 10^5$	0·9	7·6	P_3	—	—
P_4	$8{\cdot}6 \times 10^6$	1·4	2·6	P_4	$1{\cdot}5 \times 10^6$	7·9
S		93·1	25·3	S	—	27·1
Total recovery (%)		98·2	99·2	—	—	—
No. of experiments		3	4	—	—	—

* Results calculated from data of Hokin (1955).
PTR: percentage total recovery.

recovered from fractions P_2 and P_3; this loss was generally matched by an increase in activity of the supernatant (Table 7). The increased solubilization of the lysosomal enzymes probably meant that the lysosomes were more readily disrupted as a result of membrane changes produced by pilocarpine.

An interesting aspect of these experiments was that compared with its effect on other intracellular enzymes pilocarpine caused a preferential discharge of amylase and kallikrein into saliva. Their ratio of activity in the saliva (2·3) resembled that in the supernatant (3·1), indicating a similar rate of secretion. In contrast, little succinic dehydrogenase, β-glucuronidase and acid phosphatase was secreted into saliva (Text-fig. 3).

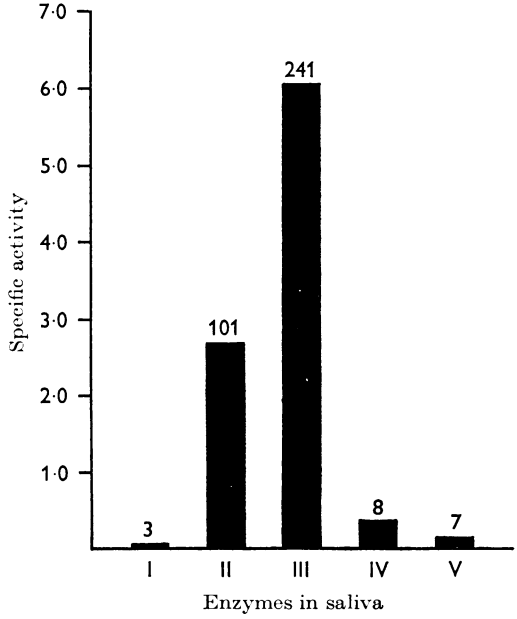
TABLE 7. Effect of pilocarpine on the distribution of intracellular enzymes

Fraction	Kallikrein		Succinic dehydrogenase		β-Glucuronidase		Acid phosphatase		Amylase	
	PTR	RSA	PTR	RSA	PTR	RSA	PTR	RSA	PTR	RSA
P_1	31·9 ± 3·8	3·98	13·4 ± 5·4	1·71	7·2 ± 3·7	0·95	8·1 ± 1·2	0·99	1·4 ± 0·2	0·17
P_2	28·0 ± 6·0	2·64	32·1 ± 1·3	3·04	15·3 ± 1·8	1·43	12·7 ± 1·3	1·19	5·8 ± 0·6	0·55
P_3	9·9 ± 1·9	0·84	31·5 ± 2·7	2·66	21·6 ± 1·6	1·81	11·6 ± 1·4	0·97	2·3 ± 0·3	0·19
P_4	1·4 ± 0·7	0·06	15·1 ± 2·9	0·76	12·5 ± 2·9	0·62	19·0 ± 2·1	0·95	1·3 ± 0	0·06
S	28·3 ± 9·2	0·58	7·4 ± 3·8	0·14	42·7 ± 3·8	0·87	49·5 ± 1·9	1·01	88·8 ± 0·5	1·82
Total recovery (%)	82·3	—	73·5	—	101·4	—	81·8	—	78·0	—

PTR: percentage total recovery. RSA: relative specific activity.
Mean results of three experiments.

Localization of kallikrein in sucrose density-gradients

Particulate kallikrein was clearly differentiated from the mitochondrial succinic dehydrogenase and the lysosomal β-glucuronidase in sucrose density-gradients, thereby confirming the results obtained by differential centrifugation. Although the distribution of acid phosphatase was undoubtedly different it was not as effectively separated from kallikrein as the other enzymes (Table 8).



Text-fig. 3. Pilocarpine induced secretion of the intracellular enzymes in saliva. I = Succinic dehydrogenase; II = kallikrein; III = amylase; IV = acid phosphatase; V = β-glucuronidase.

The figure on each histogram represents the activity of the enzyme in saliva expressed as a percentage of the activity in the homogenate and is the mean of three experiments.

The kallikrein containing particles consistently equilibrated between 1·5 and 1·9 M sucrose (Table 9). A precise localization was attempted by first centrifuging P_2 on 1·6 M sucrose; the precipitate was refloated and centrifuged on a gradient extending from 1·6 to 1·85 M sucrose at $10{\cdot}51 \times 10{\cdot}6\ g$ min. The particles associated with kallikrein were almost entirely distributed from 1·7 to 1·8 M sucrose (Table 10).

TABLE 8. Distribution of intracellular enzymes on sucrose-density gradient

Sucrose molarity P_2 subfraction	Kallikrein		Succinic dehydrogenase		β-Glucuronidase		Acid phosphatase		Protein	
	1	2	1	2	1	2	1	2	1	2
0·7	8·4	5·0	3·7	6·6	8·0	12·6	9·5	7·4	13·3	9·5
1·1 1·2	2·7	3·6	7·1	5·0	30·8	14·2	19·6	9·5	21·6	5·2
1·3 1·4	8·7	1·9	61·9	24·9	43·6	35·2	26·5	22·4	28·3	12·1
1·5 1·6	25·7	26·1	19·3	42·1	8·4	19·6	15·4	19·7	14·1	20·2
1·7 1·8	54·2	68·1	7·8	21·2	9·2	18·2	28·8	40·9	22·5	52·7
Total recovery (%)	121·1	109·8	109·6	70	121·6	115·5	94·5	132·7	98·1	102·5

1: $1{\cdot}47 \times 10^6$ *g* min; 2: $10{\cdot}51 \times 10^6$ *g* min.
Results expressed as percentage of total recovery.

Microscopy of the kallikrein containing particles

The heavy mitochondrial or zymogen fraction (P_2) was layered on 1·6 M sucrose and centrifuged at $1{\cdot}47 \times 10^6$ *g* min. The precipitate (P_2p) contained a substantial amount of the kallikrein activity but much less of succinic dehydrogenase, β-glucuronidase and acid phosphatase (Table 11)

TABLE 9. Distribution of kallikrein on sucrose density-gradient

Sucrose molarity	PTR	RSA	Sucrose molarity	PTR	RSA	Sucrose molarity	PTR	RSA
0·7	8·4	0·63	0·7	5·0	0·52	0·7	12·3	0·96
1·1–1·2	2·7	0·12	1·1–1·2	3·6	0·69	1·2–1·3	4·4	0·74
1·3–1·4	8·7	0·30	1·3–1·4	1·9	0·15	1·4–1·5	11·0	0·42
1·5–1·6	25·7	1·82	1·5–1·6	26·1	1·29	1·6–1·7	42·4	2·94
1·7–1·8	54·2	2·40	1·7–1·8	68·1	1·29	1·8–1·9	29·8	0·72
Total recovery (%)	121·1	—	—	109·8	—	—	138·5	—
g min	1	—	—	2	—	—	2	—

1: $1{\cdot}47 \times 10^6$ *g* min; 2: $10{\cdot}51 \times 10^6$ *g* min.
PTR: percentage total recovery. RSA: relative specific activity.

TABLE 10. Distribution of kallikrein on sucrose density-gradient after separation of P_2 on 1·6 M sucrose at $10{\cdot}51 \times 10^6$ *g* min

Sucrose molarity	P_2p %	Sucrose molarity*	P_2p %
1·6	4·8	1·60	3·7
1·70	19·1	1·70	28·2
1·75	24·7	1·75	11·9
1·80–1·85	7·2	1·80	12·1
		1·85	3·1
P_2p activity (%)	55·9	—	59·4

P_2p %: kallikrein activity expressed as a percentage of the activity in the precipitate of P_2 fraction.

* Mean results of two experiments.

TABLE 11. Separation of the heavy mitochondrial fraction P_2 on 1·6 M sucrose

Enzymes	P_2 precipitate PTR	P_2 precipitate RSA	P_2 supernatant PTR	P_2 supernatant RSA	Total recovery (%)
Kallikrein	60·3 ± 8·9	3·28	39·6	0·46	102·1
Acid phosphatase	22·7 ± 1·2	1·21	77·7	0·95	80·0
β-Glucuronidase	10·9 ± 1·4	0·55	89·0	1·10	100·3
Succinic dehydrogenase	10·4 ± 7·8	0·54	89·4	1·10	74·9

Mean results of three experiments
PTR: percentage of total recovery. RSA: relative specific activity.

P_2p was examined by ordinary light and phase contrast microscopy. The sediment was generally packed with particles approximately 0·35–0·7 μ in diameter (Pl. 1). They were slightly smaller than pancreatic zymogen granules which range from 0·5 to 1·5 μ (Hokin, 1955; Siekevitz & Palade, 1958*a*). The particles were almost entirely basophilic when stained with haematoxylin and eosin. They reacted positively to P.A.S.; the intensity of the reaction was unaffected by diastase, signifying an absence of glycogen in the fraction and probably the presence of glycoprotein and mucin. Smears stained with Janus Green showed only a few positive granules and staining with Oil Red O indicated that little neutral fat was present. Although kallikrein was associated with these basophilic particles the possibility of its localization in granules smaller than the limit of resolution of the light microscope cannot be excluded in this study.

Lobules of acinar cells with a strongly positive P.A.S. reaction were seen in sections of the unhomogenized gland.

DISCUSSION

No clear association was demonstrated between kallikrein and the mitochondrial enzyme succinic dehydrogenase either on differential centrifugation or in sucrose density-gradients. Since succinic dehydrogenase is specific to the mitochondrion it is unlikely that kallikrein is a mitochondrial enzyme (cf. Whittaker, 1965).

The sedimentation properties of β-glucuronidase, acid phosphatase, ribonuclease and uricase were also different from those of kallikrein. This finding and comparison with the data of Duve *et al.* (1955) on the intracellular location of lysosomal acid hydrolysases suggests that kallikrein is probably held in separate particles. Because of the enzymic heterogeneity of lysosomes a hidden association with the kallikrein containing particles could not readily be excluded from the differential centrifugation experiments (Beaufay, Jacques, Baudhuin, Sellinger, Berthet & Duve, 1964). However, in sucrose density-gradient experiments kallikrein was completely differentiated from β-glucuronidase and adequately differentiated from acid phosphatase.

Adsorption to particles during homogenization could account for the localization of kallikrein in the nuclear P_1 and heavy mitochondrial P_2 fractions. This is unlikely because when fraction P_2 was subjected to density gradient analysis kallikrein was consistently recovered from the lower end of the column between 1·6 and 1·8 M sucrose. Kallikrein would have been localized in the top fractions had its association with the P_2 fraction particles been due to simple adsorption during cell fragmentation. The results of the density-gradient experiments fully confirm that kallikrein is associated with particles more dense than lysosomes or mitochondria.

The particles in the P_2 sediment with substantial kallikrein activity stained densely basophilic. They also stained positively with P.A.S. Similar P.A.S. staining was shown by the mucus-secreting acini of the unhomogenized gland. The question whether kallikrein is contained in the mucus-secreting cells of the guinea-pig submaxillary gland cannot be answered by the present experiments and requires further investigation.

Pancreatic cell particles containing the precursors of the digestive enzymes, chymotrypsin and trypsin, have been localized mainly in the nuclear and zymogen or heavy mitochondrial fractions (Siekevitz & Palade, 1958*a*). Since the *g* force applied in the present experiments was comparable to that used for pancreatic tissue, the consistently high kallikrein activity found in the P_1 and P_2 fractions strongly suggests that kallikrein may be held in a similar type of particle.

Further, pancreatic zymogen granules containing digestive enzymes have been shown to discharge into pancreatic juice in response to the secretory stimulus of pilocarpine (Kugler, Levin, Martin & Sneddon, 1967). A similar phenomenon was observed in the present study. In response to pilocarpine stimulation kallikrein and the digestive enzyme amylase were discharged into saliva, to the exclusion of the mitochondrial and lysosomal enzymes.

Although there is no experimental proof that kallikrein has an intracellular digestive role similar to the lysosomal acid hydrolysases, such a view cannot be entirely excluded. Nevertheless, the present evidence so far suggests an extracellular role and supports an exocrine secretion of kallikrein by the submaxillary gland (Schachter, 1960; Bhoola & Ogle, 1966).

I am particularly grateful to Mr R. P. Gould for carrying out the histology. I wish to thank Miss D. A. Watson, Miss A. E. Buckley and Mr M. Sitton for able technical assistance.

REFERENCES

Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O. Z., Berthet, J. & Duve, C. de (1964). Tissue fractionation studies. Resolution of mitochondrial fractions from rat liver into three distinct populations of cytoplasmic particles by means of density equilibration in various gradients. *Biochem. J.* **92**, 184–205.

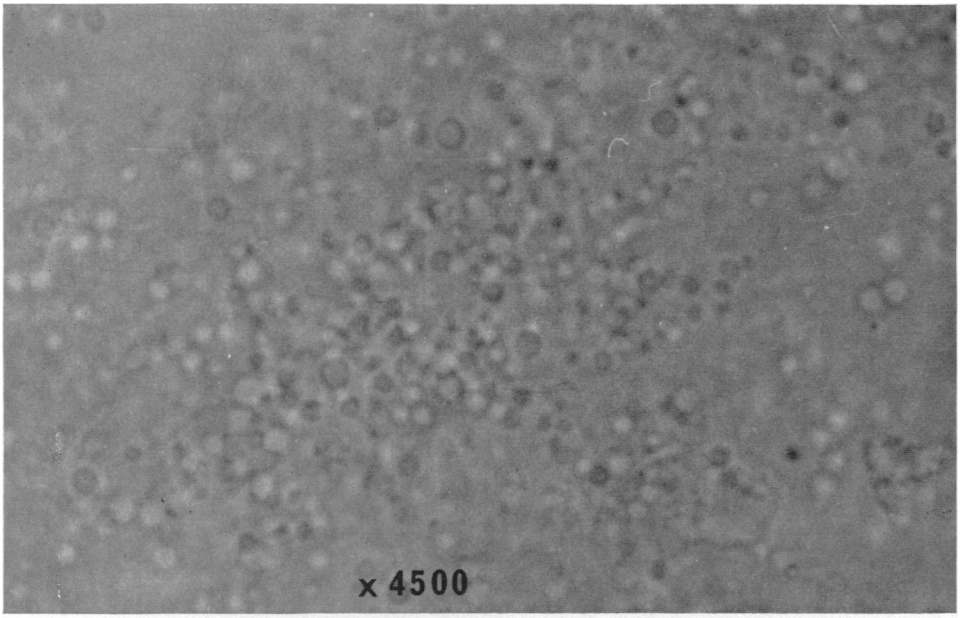
Berthet, J. & Duve, C. de (1952). Tissue fractionation studies. I. The existence of a mitochondrial-linked, enzymatically inactive form of acid phosphatase in rat-liver tissue. *Biochem. J.* **50**, 174–181.

Bhoola, K. D. & Ogle, C. W. (1966). The subcellular localization of kallikrein, amylase and acetylcholine in the submaxillary gland of the guinea-pig. *J. Physiol.* **184**, 663–672.

Duve, C. de, Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* **60**, 604–617.

Hokin, L. E. (1955). Isolation of zymogen granules of dog pancreas and a study of their properties. *Biochim. biophys. Acta* **18**, 379–388.

Holdstock, D. J., Mathias, A. P. & Schachter, N. (1957). A comparative study of kinin, kallidin and bradykinin. *Br. J. Pharmac. Chemother.* **12**, 149–158.



K. D. BHOOLA

(*Facing p.* 444)

KERR, M. H. & LEVY, G. A. (1951). The preparation and properties of β-glucuronidase. *Biochem. J.* **48**, 209–216.

KUGLER, J. H., LEVIN, R. J., MARTIN, B. F. & SNEDDON, VALMA (1967). The effects of 5-fluorouracil on the storage and secretion of pancreatic lipase and zymogen granules. *J. Physiol.* **190**, 42*P*.

LAURENT, B. & NORBERG, B. (1960). Phosphatase assay in serum with *p*-nitrophenyl phosphate as substrate. *Scand. J. clin. Lab. Invest.* **12**, 154–157.

LOWRY, O. H., ROSEBURGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

SCHACHTER, M. (1960). In *Polypeptides which affect Smooth Muscles and Blood Vessels*, ed. SCHACHTER, M., pp. 232–246. London: Pergamon.

SIEKEVITZ, P. & PALADE, C. E. (1958*a*). A cytochemical study on the pancreas of the guinea-pig. I. Isolation and enzymatic activities of cell fractions. *J. biophys. biochem. Cytol.* **4**, 203–217.

SIEKEVITZ, P. & PALADE, C. E. (1958*b*). A cytochemical study on the pancreas of the guinea-pig. II. Functional variations in enzymatic activity of microsomes. *J. biophys. biochem. Cytol.* **4**, 309–318.

WHITTAKER, V. P. (1959). The isolation and characterization of acetylcholine containing particles from brain. *Biochem. J.* **72**, 694–706.

WHITTAKER, V. P. (1965). The application of subcellular fractionation techniques to the study of brain function. In *Prog. Biophys. & molec. Biol.* **15**, 39–96.

EXPLANATION OF PLATE

Photomicrograph of fresh unstained smear of P_2 sediment prepared on albumenized slide, fixed with 10% neutral formal saline and examined by light microscopy. Magnification $\times 4500$. The major P_2 kallikrein activity was associated with similar particles.