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THE SUBCELLULAR LOCALIZATION OF KALLIKREIN, AMYLASE AND ACETYLCHOLINE IN THE SUBMAXILLARY GLAND OF THE GUINEA-PIG

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SUMMARY

1. The subcellular distribution pattern of kallikrein was compared with that of amylase and acetylcholine in submaxillary gland homogenates of the guinea-pig.

2. Kallikrein was located in particles which sedimented in the nuclear (46%) and mitochondrial (17%) fractions. These particles were probably similar if not identical to zymogen granules.

3. The above observations would support a digestive role for salivary kallikrein.

INTRODUCTION

Salivary kallikrein is one of a group of enzymes which release the vasoactive peptide, kallidin, from specific protein substrates (Werle & Roden, 1936; Werle & Berek, 1948). Soon after it was discovered, Ungar & Parrot (1936) suggested that salivary kallikrein might be the mediator for the cholinergic nerve induced vasodilation in the submaxillary gland. This view has been strongly supported by the experimental work of Hilton & Lewis (1955*a*, *b*, 1956; Lewis, 1959; Hilton, 1960), who concluded that both cholinergic and adrenergic vasodilation in this organ was produced by kallikrein. Recently much evidence has accumulated which does not support this hypothesis (Bhoola, May May Yi, Morley & Schachter, 1962; Beilson, Schachter & Smaje, 1965; Bhoola, Morley, Schachter & Smaje, 1965). The physiologic function of kallikrein remains unknown. Knowledge of its subcellular localization may prove important in establishing its physiologic role and the present experiments were therefore designed to compare the subcellular distribution of kallikrein with that of the digestive enzyme, amylase, and the neurochemical transmitter, acetylcholine.

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METHODS

Preparation of homogenates

Guinea-pigs were unfed for 12 hr, killed by a blow on the head and exsanguinated. For each experiment, submaxillary glands from eight to twelve animals (weighing 300–650 g) were dissected, rinsed in ice-cold 0·32 M sucrose, dried on filter paper and weighed (pair weight 300–800 mg). The glands were cut into small pieces, pooled and homogenized at 4°C in 0·32 M sucrose with an M.S.E. homogenizer. Homogenization was carried out at 14,000 r.p.m. for 7·5 min. The concentration of fresh gland tissue in the homogenate ranged from 100 to 250 mg/ml.

Differential centrifugation

Separation of the various fractions was carried out in the Spinco-Model L preparative centrifuge (Fig. 1). Centrifugation was started when the chamber temperature had reached 0–2° C. Rotor no. 30 was used to bring down the nuclear fraction and rotor no. 50 for the remaining fractions. The gravitational effect was calculated as the product of the force and the spinning time in minutes and expressed in *g* min.

Histology

Fresh smears of the homogenate and its particulate subfractions were stained with haematoxylin and eosin, Janus green and Heidenhain's stain and examined by light microscopy.

Assay of enzymes

1. *Succinic dehydrogenase.* Succinic dehydrogenase was measured manometrically by the Warburg micro-spirometer method (Whittaker, 1959). The hydrogen acceptor was 0·25 M-$K_3Fe(CN)_6$ in 0·023 M-$NaHCO_3$ (solution *A*). The medium (solution *B*) contained 0·14 ml. of 2 M-$MgCl_2$, 0·5 ml. of 0·1 M ethylene diamine tetra-acetic acid 1·0 ml. of 1 M phosphoric acid, 6·0 ml. of 0·3 M succinic acid and 4·0 ml. of 0·27 M-$NaHCO_3$. The pH of this mixture was first adjusted to 7·4 before adding the $NaHCO_3$. The final volume was made up to 30 ml. with distilled water. Solution *A* (0·2 ml.) was put into the side arm of the Warburg flask. Solution *B* (1·8 ml.) and 1·0 ml. of the fraction containing the enzyme (or an appropriate volume in the case of the final supernatant) were pipetted into the main compartment of the flask. For controls 1·0 ml. of 0·32 M sucrose was used instead of the fraction; controls without succinic acid were also used.

Gassing of the flasks with 5 % CO_2–95 % N_2 mixture was carried out at room temperature (20° C) for 15 min. The vessels were next placed into the water bath at 37° C. A further 15 min were allowed for equilibration and then the content of the side arm was tipped into the main compartment. The amount of CO_2 produced in μl. was taken as a measure of enzyme activity and recordings were made at 6 and 35 min; activity in each fraction was expressed as μl. of CO_2/g of fresh gland tissue.

2. *Kallikrein.* Kallikrein activity was measured by its ability to release kallidin from both heated (Holdstock, Mathias & Schachter, 1957) and unheated dialysed dog plasma. Assays were carried out using the isolated guinea-pig ileum. The various fractions were re-suspended in Mg-free Tyrode solution before assay. The original homogenate was match-assayed against the subfractions using volumes of enzyme which gave comparable concentration of gland tissue. Activity of each fraction was expressed as a percentage of the activity in the original homogenate. For qualitative confirmation of the results the isolated rat duodenum preparation and the dog's blood pressure were used.

The guinea-pig ileum preparation was suspended in Mg-free Tyrode solution (%: NaCl, 0·8; KCl, 0·02; $CaCl_2$, 0·02; NaH_2PO_4, 0·005; $NaHCO_3$, 0·1; glucose 0·1) at 35° C. Mepyramine (10^{-8} g/ml.) and atropine (10^{-8} g/ml.) were added to the bath fluid before each test. The rat duodenum preparation was suspended in atropinized (10^{-8} g/ml.), Mg-free Tyrode solution with the bath temperature at 30° C. Both preparations were bubbled with 95 % O_2–5 % CO_2

mixture. The type of bath was similar to that described by Schachter (1956). Contractions were recorded on a smoked drum with a frontal writing lever.

In vivo kallikrein activity was determined on the anaesthetized (Nembutal 60 mg/2·28 kg), dog's blood pressure which was recorded from carotid artery. Constant volume injections were made through the femoral vein.

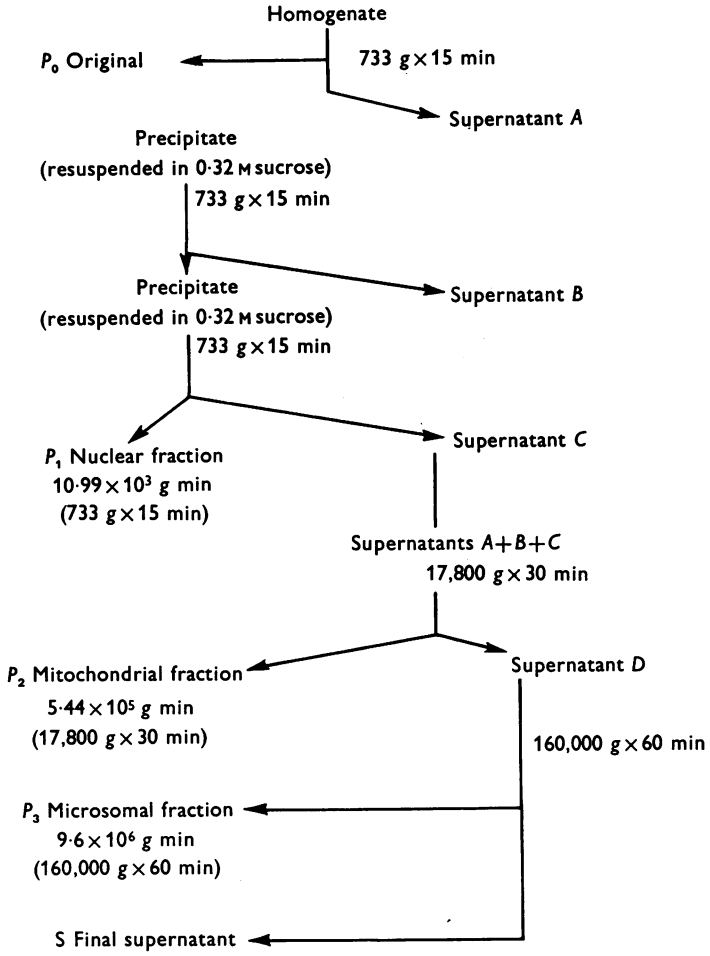

Fig. 1. Differential centrifugation.

3. *Amylase.* Amylase was measured by its ability to reduce the turbidity of a 1 % starch solution and estimated spectrophotometrically (Lange spectophotometer 2 M and galvanometer; wave-length 560 mμ, slit width 0·5 mm, temperature 37° C). The change in transmission was related to enzyme concentration. With this method the total recovered activity was always far in excess of 100 %. Siekevitz & Palade (1960) encountered a similar difficulty with pancreatic amylase, for which they could find no satisfactory explanation. We next tried estimating amylase by the iodine titration method, this proved suitable because it gave a total recovery nearer 100 %. The results of the two methods were, however, qualitatively similar.

Amylase activity was measured by adding 1·0 ml. of the homogenate or its subfractions

to a medium consisting of 5·0 ml. of 1 % soluble starch solution, 2·0 ml. of 1 % NaCl and 2 ml. of phosphate buffer (pH 6·9). Zero time was taken from the moment the homogenate containing the enzyme was added to the medium and incubated at 38° C in a water-bath. Incubated mixture (0·05 ml.) was taken every 1 min and added to a test tube containing 2·0 ml. of iodine solution (1·0 ml. of 0·01 N iodine solution diluted in 250 ml. distilled water). The end-point of the titration (achromic point) was noted in min. Under these conditions the achromic point of 5·0 ml. of 1 % soluble starch digested by 1 u. of amylase (Diastase) was 10 min. On each sample three to five estimations were carried out and the mean derived. The units of activity in each fraction was calculated by dividing the achromic point by 10 and expressing activity as u./g of fresh gland tissue.

Acetylcholine estimation

Acetylcholine was assayed on the toad's rectus abdominus muscle (*Bufo melano stictus*). The rectus muscle was suspended in eserinized (3×10^{-5} g/ml.), Ringer solution (% NaCl, 0·75; KCl, 0·015; $CaCl_2$, 0·02; NaH_2PO_4, 0·001; $NaHCO_3$, 0·025; Glucose, 0·1) in a 1·5 ml. bath at room temperature (20° C) and bubbled with 99% O_2–1 % CO_2 mixture. Sensitivity of the muscle to acetylcholine was considerably enhanced by adding 0·25 ml. of 20 % ethyl alcohol in Ringer solution before each test. Acetylcholine in final concentrations of $1{\cdot}5 \times 10^{-9}$ g/ml. could readily be detected by this method.

Free acetylcholine was measured by assaying the homogenate and its subfractions directly without any previous treatment. To obtain the total acetylcholine content of each sample, the bound ester was first released by acidification with HCl to pH 4 and heating in a boiling water-bath for 10 min. The pH of the heated, acidified fraction was adjusted with NaOH to 6·5 before assay. The values for bound acetylcholine were obtained by subtracting the free from the total. The non-acetylcholine activity in each fraction was recorded after inactivating the sample by heating at pH 13 for 10 min in a boiling water-bath and also after curarization (+)-tubocurarine) of the muscle. Acetylcholine activity was expressed in terms of μg/g of fresh gland tissue.

Nitrogen estimation

Protein nitrogen was measured by the micro-Kjeldahl method. Three separate estimations were carried out on each fraction.

Expression of activities

The amount or activity of succinic dehydrogenase, nitrogen, amylase and acetylcholine was calculated/g of fresh gland tissue and expressed as a percentage of total recovery (PTR). In the case of kallikrein, activity in each fraction was calculated as a percentage of the original and then expressed as a percentage of total recovery. The relative specific activity (RSA) was calculated by dividing the percentage activity (PTR) by the nitrogen content of the same fraction expressed as a percentage of the total recovered nitrogen (Whittaker, 1959).

Drugs and other materials. Acetylcholine was used as chloride and its doses expressed in terms of the base. Atropine was used as sulphate, mepyramine as maleate, eserine as hydrochloride and (+)-tubocurarine as chloride; their doses are expressed in terms of the salts.

Synthetic bradykinin was supplied by Sandoz Ltd. and Diastase (amylase) by Hopkins Ltd.

Sörensen's phosphate buffer (6·9); 44·6 ml. of M/15–KH_2PO_4 (9·08 g KH_2PO_4/l.) added to 55·4 ml. of M/15-Na_2HPO_4 (11·88 g $Na_2HPO_4.2H_2O$/l.).

Starch solution (1 %) was prepared by boiling distilled water and slowly adding soluble starch (Merck). The solution was stirred continuously and on moderate cooling two drops of pine oil (preservative) were added. The final volume was made up to 100 ml. and the solution kept at 4° C.

RESULTS

Distribution of succinic dehydrogenase

Both the homogenization and fractionation procedures were controlled by estimating succinic dehydrogenase activity in the subfractions of the homogenate. Since this oxidative enzyme is entirely located in the mitochondria, the conditions for homogenization and centrifugation were worked out so that the major amount of succinic dehydrogenase was contained in the subfraction with the sedimentation property and histological appearance of mitochondria (Table 1). In our experiments the mitochondrial fraction P_2 was precipitated at $5{\cdot}44 \times 10^5$ g min. Histologically

TABLE 1. Succinic dehydrogenase activity

	Measurement at 6 min			Measurement at 35 min		
Fraction	μl. CO_2	PTR	RSA	μl. CO_2	PTR	RSA
P_0	114	95·0	0·91	400	90·7	0·87
P_1	22	18·3	1·63	45	10·2	0·91
P_2	84	70·0	3·41	360	81·6	3·98
P_3	4	3·3	0·25	8	1·8	0·14
S	10	8·3	0·15	28	6·3	0·11

Total recovery 105·3 %. PTR: percentage of total recovery.
Mean result of three experiments. RSA: relative specific activity.

it appeared fairly homogeneous. The nuclear fraction P_1 was precipitated at $10{\cdot}99 \times 10^3$ g min. It was washed twice with 0·32 M sucrose in order to minimize contamination by small sized particles, but in spite of this procedure it lacked homogeneity. It contained 18·3 % of the total succinic dehydrogenase activity, and histologically comprised nuclei, mitochondria and tissue fragments. The microsomal fraction P_3 was separated from the final supernatant S by centrifugation at $9{\cdot}6 \times 10^6$ g min. The centrifugal force was increased considerably (P_3: 3×10^6 g min, Hebb & Whittaker, 1958) in order to ensure a more complete separation of microsomal particles from the final supernatant (Siekevitz & Palade, 1958*a*). The small percentage of succinic dehydrogenase in the final supernatant was probably due to dissolved enzyme.

Distribution of nitrogen

The concentration of protein nitrogen was highest in the final supernatant (55·3 %) and was found in decreasing amounts in the mitochondrial (20·5 %), microsomal (13 %) and nuclear (11·2 %) fractions (Table 2). The protein nitrogen recovered in the mitochondrial samples compares satisfactorily with the finding in hepatic mitochondria (20–25 %), but contrasts with the much lower amount (6–12 %) reported for the pancreas (Siekevitz & Palade, 1958*a*).

Localization of kallikrein

Before the subcellular fractions were assayed for kallikrein activity they were resuspended in Tyrode solution in order to reduce the final sucrose concentration from 0·32 M to approximately 0·05 M. Since the membrane of subcellular particles is known to disintegrate under these conditions (Hokin, 1955) it was not possible to establish whether free or bound kallikrein was estimated in our experiments.

TABLE 2. Estimation of protein nitrogen

Fraction	Fresh tissue (mg/g)	PTR
P_0	34·5 ± 1·8	104·2
P_1	3·7 ± 0·6	11·2
P_2	6·8 ± 0·6	20·5
P_3	4·3 ± 0·8	13·0
S	18·3 ± 1·0	55·3

Total recovery 95·9 %. PTR: percentage of total recovery.
Mean result of five experiments.

TABLE 3. Kallikrein activity

Fraction	P_0 (%)	PTR	RSA
P_1	82·7 ± 6·9	46·1	4·1
P_2	30·8 ± 6·2	17·2	0·84
P_3	0	0	0
S	66·0 ± 10·9	36·8	0·65

Total recovery 179·5 %. PTR: percentage of total recovery.
Mean result of four experiments. RSA: relative specific activity.

Kallikrein activity was divided between the nuclear fraction (46·1 %) and the final supernatant (36·8 %). Moderate amounts were detected in the mitochondrial (17·2 %) but none in the microsomal samples (Table 3). A similar distribution pattern was obtained when the kallikrein was assayed on the dog's blood pressure or the isolated rat duodenum preparation.

Localization of amylase

Most of the amylase activity of the original homogenate was recovered from the final supernatant (96·2 %). Minimal amounts were detected in the mitochondrial (1·8 %), nuclear (1·2 %) and microsomal (0·9 %) fractions (Table 4). In the present experiments only free amylase was estimated; no attempt was made to treat the samples with substances like adenosine triphosphate to release bound amylase (Siekevitz & Palade, 1960).

Localization of acetylcholine

Almost all of the free acetylcholine was recovered from the supernatant; none was present in fractions P_1, P_2 and P_3 (Table 5). Whereas only 10% of the free acetylcholine was lost on fractionation, at least 30% of bound activity was not recovered. Most of the bound acetylcholine, released by treatment with HCl, was located in the nuclear fraction (79·7%).

TABLE 4. Amylase activity

Fraction	Amylase u./g fresh tissue	PTR	RSA
P_0	1731 ± 180	105·1	1·01
P_1	19·3 ± 1·9	1·2	0·11
P_2	29·2 ± 3·4	1·8	0·09
P_3	14·6 ± 2·3	0·9	0·07
S	1583 ± 155	96·2	1·74

Total recovery 95·1%. PTR: percentage of total recovery.
Mean result of three experiments. RSA: relative specific activity.

TABLE 5. Estimation of acetylcholine

	Fresh tissue (μg/g)			PTR			RSA		
Fraction	Total	Free	Bound	Total	Free	Bound	Total	Free	Bound
P_0	1·366 ± 0·06	0·601 ± 0·07	0·766 ± 0·02	128	110·5	146·5	1·23	1·06	1·41
P_1	0·417 ± 0·05	0	0·417 ± 0·05	39·1	0	79·7	3·49	0	7·12
P_2	0	0	0	0	0	0	0	0	0
P_3	0	0	0	0	0	0	0	0	0
S	0·650 ± 0·04	0·544 ± 0·04	0·106 ± 0·09	60·9	100	20·3	1·10	1·81	0·37
Total recovery	78·4%	90·5%	68·4%	—	—	—	—	—	—

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

DISCUSSION

Our results suggest that the major kallikrein activity is held in some particulate form, probably in vesicles with sedimentation properties similar to those of zymogen granules. In the usual fractionation scheme zymogen or secretory granules occupy an intermediary position between the mitochondria and the heavier nuclei (Siekevitz & Palade, 1958*a*). In our experiments these particles were probably divided in varying ratios between the nuclear and mitochondrial samples, and if it is assumed that kallikrein is located in these granules then this would explain the distribution pattern we observed. Further, the absence of kallikrein in the microsomes of our animals which were unfed before fractionation raises

the possibility of it being a digestive enzyme (Siekevitz & Palade, 1958*b*, 1960). The presence of kallikrein in the final supernatant was probably the result of disintegration of a certain number of particles which occurs during fractionation, the liberated enzyme becoming solubilized. The reason for a very much greater than 100% total recovery of kallikrein is unknown. It may be explained by alteration in amounts of kallikrein inhibitor or kininase in each subcellular sample as a result of redistribution during fractionation.

Further supportive evidence for a digestive function would have been obtained if both kallikrein and amylase showed a similar subcellular localization. No firm conclusion could be reached on this point because of the small amounts of amylase detected in the subfractions. The reason for this may be that submaxillary gland amylase exists in both free and bound forms as in the pancreas (Laird & Barton, 1957), only the free form being estimated in our experiments. Siekevitz & Palade (1960) have shown that bound amylase could be released from pancreas ribonucleoprotein particles by adenosinetriphosphate and by compounds which complex such bivalent cations as Mg^{2+}. Further experiments are therefore necessary to establish whether bound amylase occurs in submaxillary cell fractions and to ascertain its subcellular localization.

We have shown that the distribution pattern of kallikrein and acetylcholine are dissimilar, indicating that they are probably held in different particles. Our results on bound acetylcholine are not in agreement with its known localization in the brain where it is held in vesicles similar in size to mitochondria (Hebb & Whittaker, 1958; Gray & Whittaker, 1960). Sucrose density gradient and electron microscopy studies need to be carried out on the subfraction P_1 before bound acetylcholine can be localized precisely. Such a study would also permit characterization of the particles containing kallikrein from those with acetylcholine.

The question of a spurious distribution pattern for these substances has always to be considered. A great deal of variation in the concentration and localization could conceivably occur depending on the feeding state of the animal. For instance, on feeding, modifications occur in the structure of microsomes and endoplasmic reticulum of pancreatic exocrine cells; intracisternal granules appear which become filled with a high concentration of digestive enzymes (Siekevitz & Palade, 1958*b*). In order to control such a variation our animals were unfed for 12 hr and as a result the microsomes were depleted of both amylase and kallikrein. A similar reduction in microsomal amylase in the pancreatic gland has been observed in starved guinea-pigs by Siekevitz & Palade (1960). Another cause of variation may be that protein released from one cell compartment during fractionation becomes adsorbed to any of the subcellular particles. Some

relocation by adsorption may occur but is probably not of sufficient magnitude to alter the distribution pattern.

Considerable experimental evidence has accumulated which challenges the view that salivary kallikrein plays a significant role as a mediator of functional vasodilation in the gland. An alternative view that it may have a digestive function has been suggested by Schachter (1960) and our results are in accord with such a view. Since the zymogen or secretory granule is a storage particle for digestive enzymes the localization of kallikrein in such vesicles would indicate a digestive function. However, before this can be firmly established it will be necessary to prepare pure zymogen granule fractions and demonstrate major kallikrein activity in them.

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