The Release of Adenosine Triphosphate Catabolites during the Secretion of Catecholamines by Bovine Adrenal Medulla

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1. AMP, adenosine, inosine and hypoxanthine were present in perfusates collected from bovine adrenal glands during periods when catecholamine secretion was evoked by injections of carbamoylcholine. 2. The molar ratio of catecholamines to ATP-catabolites present in the perfusates was similar to that of catecholamines to ATP in chromaffin granules. 3. ATP added to the perfusing medium was extensively degraded during passage through bovine adrenal glands. 4. The mechanism of catecholamine secretion is discussed.

Adrenaline and noradrenaline are stored in the chromaffin granules of the adrenal medulla with sufficient ATP to neutralize their positive charges since the molar ratio of catecholamines to ATP in the granules is approximately 4 (Hillarp, 1958; Schümann, 1958a). After insulin-induced hypoglycaemia (Carlsson, Hillarp & Hökfelt, 1957) or the injection of carbamoylcholine (Banks, 1965), both the catecholamine and adenine nucleotide contents of the gland fall below their control values, although the molar ratio of catecholamines to adenine nucleotides remains essentially constant. The decrease in adenine nucleotide content cannot be accounted for by a corresponding rise in the amounts of other purine derivatives within the gland (Carlsson *et al.* 1957). Recently Douglas & Poisner (1966a) have measured the release of AMP from perfused adrenal glands of cats which had been made to secrete both by the injection of acetylcholine and stimulation of the splanchnic nerve. Banks (1965) was unable to detect adenine nucleotides in the perfusate from stimulated bovine adrenal glands but Stjärne (1964) obtained evidence for the release of hypoxanthine under similar conditions.

The purpose of the present investigation was to characterize any ATP-catabolites that may be released, together with the catecholamines, from bovine adrenal medulla during periods of secretory activity, and to obtain information about the nature of the secretory process.

METHODS

Retrograde perfusion of bovine adrenal glands. The left adrenal gland was removed as soon after slaughter as possible and taken on ice to the Laboratory, where the surrounding fat was dissected away. After a polythene

cannula had been inserted into the adrenal vein, Tyrode solution (Ling & Smith, 1955) was allowed to enter the gland so that it became turgid. The surface of the gland was then covered with incisions which passed through the cortical tissue but did not penetrate the medulla. The perfusion rate was adjusted to 25 ml./min. in all the experiments. The Tyrode solution was gassed with O₂ + CO₂ (95:5) and maintained at 37.5°. The gland was suspended from the cannula in a glass chamber maintained at 37.5° by a water jacket, and was perfused for 30 min. before the first stimulation period to wash out any trapped blood. The medulla was stimulated by injecting 0.5 ml. of 10 mM-carbamoylcholine (British Drug Houses Ltd., Poole, Dorset) at 30 sec. intervals over a 3 min. period. Successive stimulation periods were separated by 15 min. intervals. To the perfusion medium collected from the gland into ice-cold vessels was added 60% HClO₄ to give a final concentration of about 3% (w/v).

Analysis of the perfusates. When the denatured protein had been spun down, the deproteinized solution was passed through a column of charcoal (Norit A) and the effluent was recycled once. Norit A columns were prepared by supporting 0.25 g. of Celite 545 on a sintered-glass disk of porosity 1 in a glass column 1 cm. diam. Above the Celite was layered a mixture containing 0.25 g. of Norit A and 0.75 g. of Celite 545. The columns were washed under suction with 15 ml. of 60% (w/v) HClO₄ followed by 20 ml. of water. After applying the perfusates the columns were washed with water until the washings ceased to give an acid reaction. Elution of the adsorbed material with ethanol-water-pyridine (5:4:1, by vol.) was carried out. The eluates were evaporated to dryness under reduced pressure in a rotary evaporator and any remaining pyridine was driven off with 10 ml. of aq. 0.1 N-NH₃ soln., which in turn was removed by rotary evaporation. The dried samples were stored at -15°.

Catecholamine assay. Samples of the deproteinized perfusates were adjusted to pH 6.0 with 20% (w/v) K₂CO₃ and the precipitated KClO₄ was removed by filtration. The total catecholamines in the solution were estimated by the method of Euler & Hamberg (1949).

Ion-exchange chromatography. The dried samples from

the charcoal columns were taken up in water, applied to a column of Dowex AG50W (X4) ion-exchange resin (20 cm. $\times$ 1 cm. diam.) and eluted in 400 ml. over a linear concentration gradient progressing from water to 1.5 N-HCl. The peaks were located by an LKB Uvicord ultraviolet absorptiometer, collected and had their spectra measured between 220 and 330 m μ . Peak 1 (see the Results section), which contained a mixture of AMP and inosine, was passed through Norit A and recycled twice before being eluted and dried as described above. The dried residue was dissolved in water, applied to a column of Dowex AG1 (X4) ion-exchange resin (20 cm. $\times$ 1 cm. diam.) and eluted in 400 ml. over a linear concentration gradient progressing from water to 0.5 N-HCl.

Paper electrophoresis. In the preliminary experiments the deproteinized perfusates were neutralized to pH 7.0 with 20% (w/v) K₂CO₃ and shaken with 2 g. of Al₂O₃ for 90 min. to remove catecholamines. The Al₂O₃ was filtered off and 60% (w/v) HClO₄ was added to the solutions to give final concentration 3%. The acid solutions were passed through Norit A, eluted and dried as described above. The dried residues were subjected to high-voltage electrophoresis on Whatman no. 1 paper at 40 v/cm. and 20 mA in pyridine (10 ml.), acetic acid (92 ml.), water (to 1600 ml.) buffer, pH 3.5, for 90 min. After electrophoresis the papers were dried and washed extensively with ethanol-ether-aq. NH₃ (sp.gr. 0.88) (90:10:1, by vol.) to remove pyridine. The spots were located under ultraviolet light and eluted with 0.1 N-HCl. The compounds were identified by their electrophoretic mobilities and absorption spectra.

Haemoglobin content of perfusates. Perfusates from control periods which had not been acidified were treated with sodium dithionite and coal gas to convert haemoglobin into carboxyhaemoglobin. The quantity of carboxyhaemoglobin was measured from the extinction at 420 m μ with 4 cm. pathlength cuvettes, and molar extinction coefficient 1.8×10^{-5} .

Estimation of ATP. In two experiments the ATP content of the perfusates was measured directly by using the fluorimetric method described by Greengard (1963).

RESULTS

Haemoglobin content of the perfusates. The possibility existed that purine derivatives found in the perfusates could have originated from erythrocytes washed out from the gland by the perfusing solution. Furthermore the number of such cells appearing in the perfusate may have risen during stimulation periods. To test this, the haemoglobin contents of perfusates collected during control and stimulation periods were measured. The concentration of haemoglobin was always less than 20 μ g./ml. and did not differ significantly in the two periods. Contamination by sufficient blood to yield a haemoglobin concentration 20 μ g./ml. could account for less than 1% of the ATP-catabolites found in the perfusates after stimulation of the adrenal medulla with carbachol (see Hill & Mills, 1961).

Preliminary analysis of perfusates. During 3 min. control periods the mean catecholamine content of the perfusates was 0.4 ± 0.15 μ mole and during

Fig. 1. Absorption spectra of deproteinized perfusion media. Curve 1: absorption spectrum of deproteinized perfusion medium collected during a control period. Curve 2: absorption spectrum of deproteinized perfusion medium collected during a stimulation period. Curve 3: difference spectrum obtained by subtracting curve 1 from curve 2.

3 min. stimulation periods it rose to 10.5 ± 3.3 μ -moles. After each stimulation period, the output of catecholamines from the gland declined exponentially over approx. 10 min. until the control level was re-established. Fig. 1 shows spectra given by deproteinized perfusates from control and stimulation periods together with their difference spectrum. The last two curves have absorption maxima at 274 m μ . After catecholamines had been removed from the solutions by shaking with aluminium oxide, the absorption maxima of the stimulation period perfusate and the difference spectrum shifted to 258 m μ (Fig. 2). The difference spectrum was very similar to the spectrum of adenine mononucleotides. By using the difference spectrum and molar extinction coefficient 15×10^3 at 258 m μ , a tentative calculation was made of the molar ratio of catecholamines to adenine-like compounds present during stimulation periods. The mean value for this ratio was 8.7 (Table 1). Further analysis of the perfusates indicated that the perfusates collected during the stimulation period contained a mixture of adenosine, inosine or hypoxanthine, or both (not separated) and occasionally AMP (Fig. 3). Although inosine and

Fig. 2. Absorption spectra of deproteinized perfusion media after the removal of catecholamines with Al_2O_3 . Curve 1: absorption spectrum of deproteinized perfusion medium collected during a control period and treated with Al_2O_3 . Curve 2: absorption spectrum of deproteinized perfusion medium collected during a stimulation period and treated with Al_2O_3 . Curve 3: difference spectrum obtained by subtracting curve 1 from curve 2.

hypoxanthine were not separated by high-voltage electrophoresis, both were shown to be present in the desalted perfusate by fractionation on Dowex AG 50W. The irregular appearance of AMP was, in part, a consequence of using aluminium oxide to remove catecholamines. When $1\text{ }\mu\text{mole}$ of AMP was added to 200ml. of Tyrode solution, only some 20% could be recovered in solution after shaking with 2g. of aluminium oxide for 90min. It was therefore necessary to devise a fractionation procedure that would allow the quantitative recovery of AMP, adenosine, inosine and hypoxanthine from the perfusates. The technique finally adopted was that outlined in the Methods section, which gave the following reproducible percentage recoveries: AMP (50), adenosine (95), inosine (82), hypoxanthine (102), with $1\text{ }\mu\text{mole}$ samples of each compound dissolved in 200ml. of Tyrode solution containing $10\text{ }\mu\text{moles}$ of catecholamines.

Further analysis of perfusates. Fig. 4(a) shows the elution pattern of a mixture containing $1\text{ }\mu\text{mole}$ of each of AMP, adenosine, inosine, hypoxanthine and $5\text{ }\mu\text{moles}$ of both adrenaline and noradrenaline from a column of Dowex AG 50W ion-exchange

Table 1. Catecholamine and purine derivative contents of perfusion media collected during stimulation periods

The content of purine derivatives was calculated from the difference spectra between perfusion media collected during control and stimulation periods. For the calculation, a molar extinction coefficient 15×10^3 at $258\text{ m}\mu$ was used. Each line relates to a separate experiment.

Amount released in 3 min. stimulation period (μmoles)		
Catecholamines	Purine derivatives (calculated as adenine)	Molar ratio Catecholamine: adenine
8.9	1.1	8.1
7.9	0.9	8.8
8.9	0.9	9.9
15.4	2.4	6.4
9.2	0.9	10.2

					-ve
					Adenosine
					Inosine
					AMP
					+ve
Hypoxanthine	Stimulation Period 4	Stimulation Period 1	Control Period	Standard	

Fig. 3. Tracing of electrophoretograph showing the ATP catabolites present in the perfusion media after stimulation of a bovine adrenal gland by the injection of carbamoylcholine. The perfusion media were deproteinized and treated with Al_2O_3 to remove catecholamines before being desalted on Norit A. The compounds adsorbed on Norit A were eluted with ethanol-water-pyridine (50:40:10, v/v) and evaporated to dryness. The dried material was dissolved in water and subjected to high-voltage electrophoresis on Whatman no. 1 paper at 40 v/cm . for 90 min. in pyridine-acetic acid buffer, pH 3.5.

resin. The components of peak 1 were separated by further chromatography on Dowex AG 1 and shown to be AMP and inosine. Peak 2 contained only hypoxanthine and peak 3 contained catecholamines and adenosine; the presence of the latter was revealed when the catecholamines were removed by shaking the neutralized solution with aluminium oxide. Fig. 4(b) shows the elution pattern obtained when Tyrode solution, containing the same mixture of purine derivatives and catecholamines as described above, was desalted on Norit A, eluted, evaporated to dryness and fractionated on Dowex AG 50W as described in the Methods

Fig. 4. Identification of ATP-catabolites present in perfusion media by ion-exchange chromatography on Dowex AG50W. (a) Elution pattern obtained when a mixture, containing AMP, adenosine, inosine, hypoxanthine, noradrenaline and adrenaline, was fractionated on Dowex AG50W ion-exchange resin. (b) Elution pattern obtained when Tyrode solution containing the same mixture of purine derivatives and catecholamines as described in (a) was desalted on Norit A, eluted, evaporated to dryness and fractionated on Dowex AG50W ion-exchange resin. (c) Elution pattern obtained when perfusing medium collected during stimulation periods in Expt. 7 was fractionated in the manner described for the mixture in (b). (d) Elution pattern obtained when perfusion medium collected during control periods in Expt. 7 was fractionated in the manner described for the mixture in (b).

section. The initial peak contained oxidation products of catecholamines produced under the alkaline conditions of elution from Norit A. Fig. 4(c) shows the elution pattern obtained when the stimulation period perfusate from Experiment 7 (Table 2) was treated in the same way as the Tyrode solution shown in Fig. 4(b). Peak 1 (Fig. 4c) was further fractionated on Dowex AG1 and found to contain AMP and inosine together with two minor, unidentified components. Peak 2 (Fig. 4c) contained hypoxanthine and peak 3 (Fig. 4c) was shown to contain adenosine after removal of catecholamines with aluminium oxide. Fig. 4(d)

shows the elution profile obtained from the control period perfusate in Expt. 7. The mean value for the molar ratio of catecholamines to ATP-catabolites appearing in the perfusates during stimulation periods was 5.7 (Table 2). AMP was always the major ATP-catabolite found in the perfusates but the relative contribution to the total made by each of the four, AMP, adenosine, inosine and hypoxanthine, varied from experiment to experiment.

Model experiments showed that ATP and ADP were not retained on Dowex AG50W and that any ATP or ADP present in the stimulation period perfusates would pass through the cation-exchange resin with the catecholamine oxidation products in the initial peak. In experiments in which this peak was analysed further on Dowex AG1, no ATP or ADP could be detected.

ATP injected into the perfusing solution before it entered the gland was degraded extensively during passage through the gland (Table 3). AMP, adenosine, inosine and hypoxanthine were amongst the breakdown products recovered. The overall recovery of ATP and its catabolites was 93%.

ATP in the perfusates. When adrenal glands perfused at 25ml./min. were stimulated by the injection of 2ml. of 10mm-carbachol, very small amounts of ATP could be detected in the perfusates, by using the fluorimetric method of assay. The molar ratio of catecholamines to ATP was always very high under these conditions (Table 4).

DISCUSSION

The results presented in this paper support those of Douglas & Poisner (1966a) in showing that AMP and adenosine are liberated from adrenal medullae during periods of secretory activity, but the perfusates collected during stimulation periods in the present experiments also contained inosine and hypoxanthine. The appearance of these last two compounds, which were not found by Douglas & Poisner, is probably a consequence of using a retrograde method of perfusion in which the perfusate has to pass over cut surfaces of the cortex after leaving the medulla. This would provide additional sites for the further degradation of AMP not available in more conventional methods of perfusion. The mean value for the molar ratio of catecholamines to ATP-catabolites in the perfusates after stimulation was 5.7. This molar ratio is similar to that of catecholamines to ATP found in purified chromaffin granules (Hillarp, 1958). Thus it seems reasonable to suppose that the ATP catabolites found in the perfusates are derived from the ATP of the chromaffin granules. This view is supported by the demonstration that the loss of adenine nucleotides from perfused bovine adrenal glands during periods of secretory activity

Table 2. *Release of ATP-catabolites from perfused bovine adrenal glands during control periods and after stimulation by carbamoylcholine*

In each experiment, perfusates collected during four 3 min. control periods (section A of Table) or four 3 min. stimulation periods (section B) were pooled and fractionated by ion-exchange chromatography after being deproteinized and desalted on Norit A. The glands were perfused at 25 ml./min. with oxygenated Tyrode maintained at 37.5° and were stimulated by the injection of 0.5 ml. of 10 mM-carbamoylcholine at 30 sec. intervals over 3 min. periods. Successive stimulation periods were separated by 15 min.

Amount released in four 3 min. collection periods (μmoles)								
		Catechol- amines	AMP	Adenosine	Inosine	Hypoxan- thine	Total ATP catabolites	Molar ratio Catecholamines ATP-catabolites
A.	Control							
	6	1.4	—	—	0.15	—	0.15	9.3
	7	3.7	0.10	—	0.10	0.79	0.99	3.7
	8	1.8	—	—	0.13	0.73	0.86	2.1
	Mean	2.3	0.03	—	0.13	0.51	0.67	3.4
B.	Stimulated							
	6	23.3	1.43	1.47	0.27	0.42	3.59	6.5
	7	49.8	3.24	1.80	1.20	1.38	7.62	6.5
	8	48.5	5.22	1.59	1.45	1.65	9.91	4.9
	Mean	40.5	3.30	1.62	0.97	1.51	7.04	5.7

Table 3. *Recovery of ATP and its catabolites after the injection of ATP into the perfusing medium before it entered the gland*

The perfusion rate was 25 ml./min. and the gland was maintained at 37.5°. ATP (1 mM) was injected at 30 sec. intervals over a period of 12 min. in 0.45 ml. volumes. The perfusing medium was analysed by ion-exchange chromatography as described in the text.

	Amount (μmoles)
Added to Tyrode solution entering gland	
ATP	10.6
Recovered in perfusate leaving gland	
ATP	1.2
ADP	0.4
AMP	4.3
Adenosine	1.9
Inosine	0.7
Hypoxanthine	1.4
Total ATP and catabolites recovered	9.9

is, such that the molar ratio of catecholamines to adenine nucleotides within the gland remains constant (Banks, 1965). Both of these observations are to be expected if chromaffin granules release their ATP and catecholamines simultaneously.

The omission of Ca²⁺ ions from the perfusing solution inhibits both the spontaneous and stimulated release of catecholamines (Douglas & Rubin, 1961). As Douglas & Poisner (1966a) have pointed out, the removal of Ca²⁺ ions would be expected to render the cell membrane less stable and to facilitate the efflux of compounds of low molecular weight from the cytoplasm. Indeed Woodin & Wieneke (1963) have shown that in the absence of

Ca²⁺ ions β-glucuronidase extrusion from the granules of leucocidin-treated leucocytes is inhibited, whilst the leakage of soluble cytoplasmic components is increased. These considerations render it unlikely that a cytoplasmic pool of free catecholamines is the immediate source of secreted amines and that the role of the chromaffin granules is merely to replace catecholamines lost from the cytoplasmic pool during secretion. The observed facts are most simply explained if the catecholamines and adenine nucleotides are released directly into the extracellular space from the chromaffin granules in a process that requires the influx of Ca²⁺ ions after depolarization of the cell membrane (Douglas & Poisner, 1962; Kanno, Sampson & Douglas, 1966). Electron-microscopic studies have led de Robertis & Sabatini (1960) and Coupland (1965) to the view that the membranes of the chromaffin granules fuse with the cell membrane during secretion. This would allow the contents of the granules to be liberated from the cells by a process of reversed phagocytosis similar to that described for zymogen secretion by Palade (1959), and for enzyme extrusion from leucocytes by Woodin, French & Marchesi (1963). This view is supported by the demonstration that soluble protein originating from the chromaffin granules can be detected in perfusates collected from stimulated bovine adrenal glands (Banks & Helle, 1965; Kirshner, Sage, Smith & Kirshner, 1966).

The mechanism of catecholamine secretion outlined above may be similar to that responsible for the release of noradrenaline at adrenergic nerve terminals. Both processes require Ca²⁺ ions (Burn & Gibbons, 1964) and in nerves noradrenaline is

Table 4. Presence of ATP in perfusates from bovine adrenal medullae after stimulation

Glands were perfused with oxygenated Tyrode solution at 25 ml./min. and maintained at 37.5°. Secretion was initiated by the injection of 2 ml. of 10 mM-carbamoylcholine at zero time. The ATP content of the perfusates was measured by the fluorimetric method described in the text. Two separate experiments are presented.

Time after stimulation (sec.)	Amount present in perfusates		Molar ratio Catecholamines ATP
	Catecholamines (mμmoles)	ATP (mμmoles)	
- 12 to 0	50	0	—
0 to 12	185	0	—
12 to 24	835	5	167
24 to 36	1050	10	105
36 to 48	1010	7.5	135
48 to 60	185	0	—
- 12 to 0	75	0	—
0 to 12	400	0	—
12 to 24	1050	5	210
24 to 36	1050	5	210
36 to 48	950	3.5	271

stored in synaptic vesicles together with equivalent amounts of ATP (Schümann, 1958b). Furthermore, the occurrence of miniature end-plate potentials at the junctions between adrenergic nerves and smooth muscles has been ascribed to the sub-threshold depolarization of the muscle membrane by packets of noradrenaline released spontaneously as synaptic vesicles fuse with the axon membrane and liberate their contents (see Burnstock & Holman, 1966).

Experiments have been described in this paper which show that small amounts of ATP were present in perfusates after stimulation of the medulla. The molar ratios of catecholamines to ATP were very high but agreed well with those found by Douglas & Poisner (1966a). In view of the extensive degradation of added ATP as it passed through the gland, it is not possible to say whether ATP is released from the chromaffin granules as such and is subsequently degraded, or whether it is degraded, at least in part, during the secretory process. Douglas & Poisner (1966b) have found catecholamine to ATP ratios in perfusates as low as 11:1 when Ba²⁺ ions act as the secretagogue but it is not entirely clear how far these observations can be applied to more physiological conditions.

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