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OBSERVATIONS ON THE INTRACELLULAR GRANULES OF THE ADRENAL MEDULLA

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At a meeting of the British Pharmacological Society in January 1953 a report was made of the observation that in homogenates of the bovine adrenal medulla the bulk of the pressor activity was to be found in a fraction of granules with sedimentation properties similar to those of mitochondria (see also Welch & Blaschko, 1953).

Our observations began with an attempt to obtain homogenates of chromaffine tissue from which the pressor activity had been largely eliminated, in order to study more satisfactorily those enzyme systems concerned with the biosynthesis of noradrenaline and adrenaline. Aqueous solutions of adrenaline were quickly freed of their pressor activity by dialysis against a preparation of mushroom polyphenol oxidase, but the pressor activity of homogenates of bovine adrenal medulla was reduced only very slowly by this procedure. Thus, the presence in these homogenates of diffusion barriers which held back a major fraction of the pressor amines was suggested.

Accordingly, it was decided to study the distribution of adrenaline (and noradrenaline) in the chromaffine tissue by the use of differential centrifugation. The technique permits fairly well-defined fractions of cytoplasmic constituents to be obtained. When certain precautions are observed, for example, the use of low temperatures and of non-electrolyte media (such as sucrose solutions), separation of the fractions is made easier. Differential centrifugation had previously been found useful in this laboratory in the study of the intracellular localization of amine oxidase (Hawkins, 1952).

Most of the work to be reported here was carried out on the adrenal medulla of the ox. This species was chosen for two reasons: first, the size of the gland and the relatively defined boundary line made it easy to separate medulla from cortex, and secondly, the bovine medulla was known to contain L-DOPA-decarboxylase (Langemann, 1951), an enzyme which forms from an inactive precursor a catechol amine with pressor activity.

MATERIAL AND METHODS

Adrenal glands were obtained from freshly killed animals at the slaughterhouse and rapidly dissected. Although the time that elapsed between the death of the animal and the preparation of the homogenates was not always recorded, usually the glands were dissected in the laboratory between about 30 and 70 min after death.

The medullary tissue was weighed and rapidly cut into smaller pieces; it was then dispersed in a previously chilled, glass homogenizer. Ice-cold solutions of sucrose were used, and all subsequent operations were carried out at as near 0° C as was possible. Unless otherwise stated, the final volume of the homogenate was so adjusted that each ml. of the suspension corresponded to 0·1 g of fresh tissue.

The centrifugation procedure was not the same throughout and will be described below. In most experiments an MSE refrigerated centrifuge was used; this instrument has a 'superspeed angle-head' attachment. In some of the later experiments, in which the 'small granule' fraction was sedimented separately, a SPINCO preparative ultracentrifuge was employed. The gravitational forces attained are reported in terms of g at the bottom of the centrifuge tube.

Pressor activity of the fractions was determined on the arterial blood pressure of the spinal cat. All assays were in terms of (−)-adrenaline (base). For the determination of the total pressor activity of a fraction, an equal volume of N-HCl was added and the sample (usually the volume was 2 ml.) was frozen at −12° C. In this condition, it could be stored; but even when the assays were to be carried out immediately afterwards, the samples were first frozen, in order to ensure uniformity of treatment. Before the assay, the samples were thawed, and one-half volume of sodium phosphate buffer (0·067 M) of pH 7·4 was added. The sample was then carefully neutralized with N-NaOH, using bromothymol blue as an external indicator. A green colour was taken as the end-point; this was usually reached with slightly less than one volume of alkali; the total volume was then brought up to four times the volume of the original sample by adding a little more phosphate buffer.

The determination of protein nitrogen content was carried out on some of the fractions; a volume of 1–4 ml. was used for each determination. In order to remove the relatively large amounts of non-protein nitrogenous material, e.g. adrenaline, the following washing procedure was adopted: 10 ml. of 12% (w/v) trichloroacetic acid was added to the sample. The mixture was centrifuged at 2500 rev/min for 30–60 min in order to obtain a firm sediment. The supernatant fluid was sucked off and the precipitate was resuspended in 10 ml. of trichloroacetic acid (12%). The centrifugation was repeated and the supernatant fluid removed as before. The precipitate was then suspended in 2·0 ml. of 0·5 N-NaOH and transferred to micro-Kjeldahl flasks. Otherwise, the method did not differ from that routinely used in this laboratory (see Hawkins, 1952). Means of duplicate determinations are given in the tables. The protein values were obtained from those for the protein nitrogen by multiplication by 6·25.

EXPERIMENTAL

Distribution of pressor activity in sucrose homogenates of chromaffine tissue

The principle of the procedure employed throughout these experiments has remained unchanged, but certain modifications of detail have been introduced. A 1 in 10 homogenate of medullary tissue was prepared in ice-cold sucrose solution; the resulting homogenate was then freed of incompletely fragmented cells, of fibrous tissue and of red corpuscles by low-speed centrifugation. A *low-speed supernatant* fluid was thus obtained, an acidified sample of which was kept for assay; the remainder was centrifuged at high speed. The supernatant fluid was decanted; the sediment was resuspended, usually in the

original volume of fluid, so that 1·0 ml. of each fraction was considered as derived from 0·1 g of fresh tissue. Acidified samples of the *high-speed supernatant* fluid and of the *resuspended high-speed sediment* were retained for assay.

In the earlier experiments of this series, our principal aim was to ensure complete removal of large cellular fragments. For this reason, the preliminary low-speed centrifugation was at 950*g* for 30 min which is rather longer than is usual in the separation of cytoplasmic granules. One experiment of this kind has already been described (Blaschko & Welch, 1953). This procedure was maintained throughout the experiments which form the basis of Tables 1–3.

TABLE 1. Pressor activity of fractions obtained from homogenates of bovine adrenal medulla in 0·25 M-sucrose

(The high-speed centrifugations were at 22,000*g* for 30 min. Pressor activity in terms of μg (−)-adrenaline per ml. of fraction. The percentages (figures in parentheses) refer to the fraction from which the sample is derived, i.e. in columns *B* and *C* to column *A*, and in columns *D* and *E* to *C*.)

	1st high-speed		2nd high-speed	
Low-speed supernatant	Supernatant	Resuspended sediment	Supernatant	Resuspended sediment
A	*B*	*C*	*D*	*E*
720	135 (19)	520 (72)	20 (4)	600 (115)
1100	260 (24)	800 (73)	40 (5)	730 (91)
960	270 (28)	600 (63)	25 (4)	500 (83)
800	80 (10)	720 (90)	80 (11)	—
640	160 (25)	530 (83)	40 (8)	530 (100)
640	140 (22)	400 (63)	—	—
1000	230 (23)	800 (80)	40 (5)	800 (100)
800	170 (21)	520 (65)	—	—
625	130 (21)	500 (80)	—	—
620	100 (16)	450 (73)	—	—
840	255 (30)	670 (80)	—	—
Mean percentages	22	74	6	98

In the low-speed centrifugation some fibrous tissue and some greyish-purple coloured debris settled at the bottom of the centrifuge tube; on top of this layer a bright-red band of red blood cells was seen, and on top of this a narrow yellowish white layer which presumably was composed mainly of nuclei. The supernatant fluid, when inspected under the microscope, was essentially free of nuclei; it consisted of masses of finely granular material, partly clumped together, but for the most part dispersed and exhibiting Brownian movement.

The supernatant fluid obtained by low-speed centrifugation was re-centrifuged at higher speed; in the experiments summarized in Table 1, this centrifugation was for 30 min at 22,000*g*. The sucrose concentration was either 0·25 M (Table 1) or 0·88 M. The appearance of the supernatant fluid differed in these two media. In 0·25 M-sucrose it was almost clear, while in 0·88 M-sucrose it was opaque. This suggested that in 0·25 M-sucrose sedimentation of the

'microsomes' was largely complete, whereas in the hypertonic medium (0·88 M-sucrose) they had remained in suspension.

In the earlier experiments, the sediment was resuspended by gently rubbing it in the centrifuge tube, using a glass rod with a ball-shaped end; the suspension was filtered through a small plug of cotton-wool. In later experiments, the sediment was resuspended by agitation in a glass-stoppered measuring cylinder and the filtration through cotton-wool was omitted.

In both media most of the pressor activity had been sedimented by high-speed centrifugation. Table 1 shows that an average of 22% remained in the supernatant fluid and that 74% was recovered in the sediment. This result indicates that the two fractions accounted for the total pressor amine content of the low-speed supernatant from which they were derived.

In five of the experiments of Table 1 the resuspended high-speed sediments were again centrifuged for 30 min at 22,000g. Assays of the supernatant fluids and resedimented particles showed that only a very small fraction, about 6%, of the total pressor amine content, was present in the supernatant fraction. The remainder was again recovered in the sediment.

In the hypertonic medium (0·88 M-sucrose) the distribution of pressor activity between sediment and supernatant was similar to that observed with 0·25 M-sucrose, with a mean recovery of 80% of the total adrenaline in the high-speed sediment. This indicated that the small granules of the 'microsome' fraction, which remained suspended in the hypertonic medium, did not contain a significant part of the total adrenaline-like material. This conclusion was confirmed in experiments which will be described below.

Table 2 summarizes the results of more recent experiments in which two changes were introduced. First, the concentration of the sucrose medium was 0·32 m, which is more nearly isotonic with 'physiological' media and, secondly, duration and force of the high-speed centrifugation were reduced, in order to ensure that the bulk of the 'microsome' fraction remained suspended in the supernatant fluid. Table 2 shows that centrifugation in 0·32 m-sucrose for 20 min at 11,000g gave a distribution of adrenaline-like activity very similar to that found in the experiments shown in Table 1.

The results of these experiments can be summarized in the following manner: in homogenates of the bovine adrenal medulla in ice-cold sucrose about three-quarters of the total pressor activity was contained in a granular fraction; these granules, when resuspended in an appropriate ice-cold solution of sucrose, gave up only a very small fraction of their amine content, and most of the activity again could be sedimented by high-speed centrifugation.

Observations on sheep glands. Three similar fractionations were carried out on the medullae from sheep suprarenal glands; these glands came from animals which had been used in experiments at the Nuffield Institute for Medical Research. The medullae were separated and homogenized in 0·25 M-

sucrose. The results are shown in Table 3 with the approximate time after death at which each gland was dissected. Although in the first two experiments considerable pressor amine was found in the supernatant, in the last experiment where the glands were removed immediately after death the distribution of pressor activity between high-speed supernatant and resuspended sediment was similar to that usually found in homogenates of the bovine medulla.

TABLE 2. Pressor amine contents of fractions derived from bovine medullary homogenates in 0·32 m-sucrose

(High-speed centrifugation for 20 min at 11,000*g*. Activity in μg adrenaline per ml. of fraction. Percentages in parentheses.)

	High-speed	
Low-speed supernatant *A*	Supernatant *B*	Resuspended sediment *C*
2400	560 (23)	1445 (60)
510	125 (25)	460 (90)
560	50 (9)	535 (95)
860	245 (28)	640 (75)
680	130 (19)	435 (64)
865	240 (28)	490 (57)
1600	225 (14)	570 (36)
855	255 (30)	670 (78)
1200	340 (28)	865 (72)
1000	220 (22)	730 (73)
1280	435 (34)	1360 (106)
920	260 (28)	655 (71)
920	220 (24)	960 (104)
Mean percentages	24	76

TABLE 3. Distribution of pressor activity in homogenates of sheep suprarenal medulla in 0·25 M-sucrose

			High-speed	
Expt.	Time after killing	Low-speed supernatant (μg/ml.)	Supernatant (μg/ml.)	Resuspended sediment (μg/ml.)
1	Over 1 hr	500	390	90
2R	Approx. 1 hr	550	265	290
2L	Approx. 1 hr	580	250	260
3	Under 20 min	1120	280	935

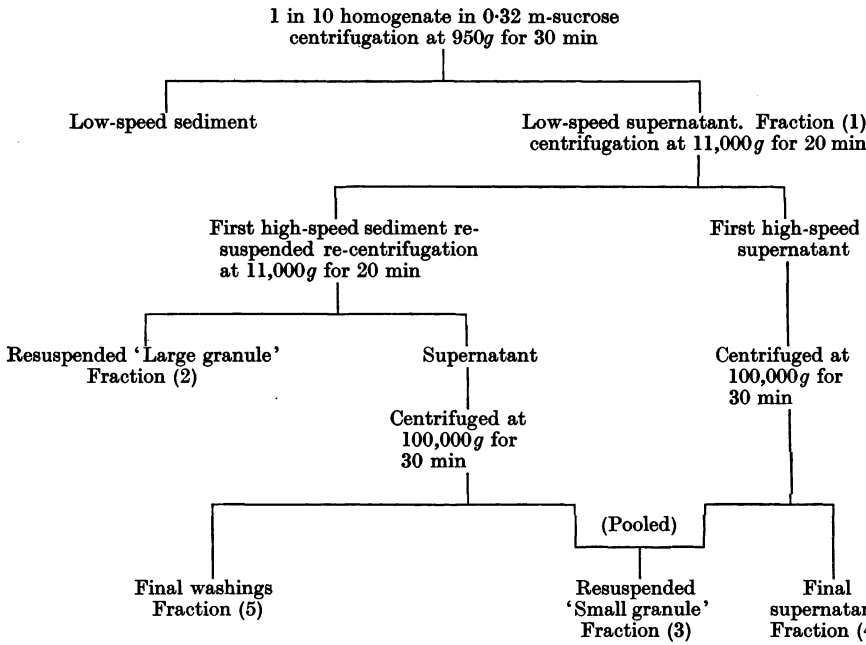
These results indicate that the post-mortem changes tended to dissociate the adrenaline from the granules. It appears that in the sheep these changes occurred more rapidly than in the ox.

Pressor amines in 'large' and 'small' granule fractions

In order to obtain direct information on the pressor amine content of the microsome, or 'small' granule fraction, we have carried the centrifugation procedure a step further. A scheme of the procedure adopted is shown in Table 4. In these experiments the homogenate and the low-speed supernatant were prepared as described before, and the latter was centrifuged at 11,000*g*

for 20 min. It was assumed that most of the microsomes were present in the supernatant fluid, but that some possibly had been sedimented with those particles resembling mitochondria in sedimentation properties. Accordingly, the resuspended sediment was recentrifuged for 20 min at 11,000g and the sediment retained as the 'large granule' fraction. The supernatant from this

TABLE 4. Scheme of centrifugation for obtaining 'small' and 'large' granule fractions of homogenates from the bovine suprarenal medulla



centrifugation was centrifuged, as was that from the first high-speed centrifugation, in the SPINCO centrifuge at 100,000g for 30 min. The sediments from these runs were resuspended together, but the supernatant fluids were kept separate. Of these different fractions, the following were examined for pressor amine content: (1) the low-speed supernatant, (2) the 'large granule' fraction, (3) the 'small granule' fraction, (4) the final supernatant fluid and, (5) the final washings. The sum of the pressor amine content of fractions (2) to (5) inclusive should account for that of fraction (1).

One complete experiment of this kind has been carried out on a 1 in 10 bovine homogenate in 0·32 m-sucrose. The result of the assays is shown in Table 5. It can be seen that 96% of the total pressor amine content of fraction (1) was accounted for. The bulk of the amine content was in the large granules; the small granules contained only 5% of the total activity.

Determination of volume of sediment. In the experiments described in this

paragraph data on the concentration of pressor amines (expressed as adrenaline) in the granules have been calculated. This calculation was based upon an estimate of the volume of sediment, together with a determination of the pressor activity.

TABLE 5. Distribution of pressor activity in fractions of bovine medullary homogenate (Expt. no. 84)

(Fractionation procedure as outlined in Table 4. Pressor activity expressed in terms of μg (−)-adrenaline per ml. of fraction. Sucrose concentration: 0·32 m.)

		μg/ml.	% of (1)
Low-speed supernatant	(1)	1170	—
Large granule fraction	(2)	840	72
Small granule fraction	(3)	56	5
Final supernatant	(4)	200	17
Final washings	(5)	20	2

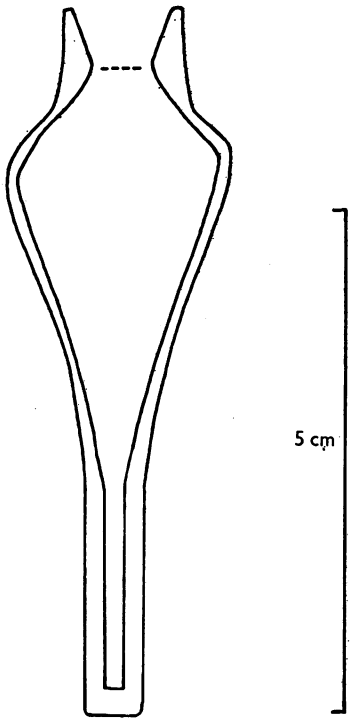


Fig. 1. Chondriocrit vessel for determination of granular volume.

For these experiments a glass vessel was used which in the following will be called a 'chondriocrit'. It was made by Mr A. Leemans of the Oxford University Glassblowing Unit. The shape of the instrument is shown in Fig. 1. The vessel had a total capacity of about 1·5 ml.; it ended in a capillary made of 'Veridia' glass precision bore capillary of 2 mm internal diameter; the bottom

of the capillary was flat. The instrument fitted into the high-speed attachment of the MSE centrifuge. It was filled with a resuspended sediment from high-speed centrifugation and centrifuged until the granules had settled at the bottom of the capillary. The upper level of the sediment was then read. Since the diameter of the capillary was known, a figure of the volume of the layer of granules could thus be obtained. The adrenaline content of the whole suspension was also determined. In this way estimates of the adrenaline concentration in the layer of granules was obtained; these are shown in Table 6. This determination gave figures which must be lower than the true values. In the calculation it was assumed that the entire volume of the sedimented granules was space in which adrenaline was dissolved. Even so, the figures given in Table 6 for the apparent adrenaline content are very high.

TABLE 6. Adrenaline concentration in medullary granules

(Determination of granular volume by use of the chondriocrit. Centrifugation as for experiments of Tables 1 and 2.)

Sucrose concentration	Adrenaline molar concentration
0·25 M	0·14
0·25 M	0·20
0·25 M	0·11
0·32 m	0·19
0·32 m	0·29

'Top' and 'bottom' layers. Inspection of the sedimented 'large granule' fraction showed that it consisted of two layers. The bottom layer appeared brown and more closely packed; the top layer had a yellowish white appearance. Often a distinct boundary line was seen between the two zones; this was also seen in the chondriocrit tubes.

In order to obtain enough material for an examination of these layers, a 1 in 10 homogenate of bovine medullary tissue in 0·32 m-sucrose was made as before and from it a high-speed sediment was prepared (see Table 4). This sediment was resuspended in a small volume of 0·32 m-sucrose, just enough to collect the sediments of all the six centrifuge tubes of the superspeed angle head centrifuge into two tubes. These two tubes, each containing 5 ml. of fluid, were re-centrifuged at 11,000*g* for 30 min. The supernatant fluid was then carefully removed by suction and the sides of the tubes were dried with filter paper. The top layer was removed with a Pasteur pipette; the bottom layer could then be similarly collected. The collected layers were well stirred, and weighed samples were taken, for determination of dry weight, for adrenaline assay and, in a number of experiments, for determination of protein nitrogen and of succinoxidase activity.

Table 7 contains the results of the adrenaline assays of the two layers, as well as data on the dry weight of the two fractions. With the exception of Expt. 88, the two layers differed considerably in percentage dry weight and in

adrenaline content. The mean figure for dry weight was 19·1% for the top layer and 26·3% for the bottom layer. In the calculation of the averages, the figures for one experiment (no. 88) have been omitted; since a clear separation of the sediment into layers was not observed in this experiment, and the sampling of the two fractions was rather arbitrary, this omission is regarded as justified. Table 7 shows that the adrenaline concentration of the top layer ranged from 5·2 to 8·4% of the dry weight, while that of the bottom layer ranged from 11·8 to 17·0%. The mean figure for the top layer was 6·1%, that of the bottom layer, 14·3%. If Expt. 88 is included the values become 6·3 for the top layer and 13·2 for the bottom layer. In other words, the adrenaline concentration in the bottom layer was more than twice that in the top layer. In Table 7, the adrenaline content has been expressed in terms of molal concentration, on the assumption that the adrenaline was uniformly dissolved in the water of the granular fraction.

TABLE 7. Dry weight and adrenaline content of 'top' and 'bottom' layers of ox medullary granules (fraction 2)

	'Top' layer			'Bottom' layer		
		Adrenaline content			Adrenaline content	
Expt. no.	Dry weight, % of wet weight	As % of dry weight	Molal concentration	Dry weight, % of wet weight	As % of dry weight	Molal concentration
59	20·0	6·8	0·09	32·0	16·0	0·41
61	18·0	5·4	0·07	25·4	12·3	0·23
62	18·6	5·2	0·07	27·1	17·0	0·35
87	19·5	5·7	0·08	25·7	12·9	0·24
88	22·1	7·3	0·11	21·2	6·4	0·10
94	18·4	5·2	0·07	24·1	11·8	0·20
95	18·9	—	—	24·4	—	—
97	20·8	8·4	0·12	25·1	16·0	0·29

Pressor amine content and protein nitrogen. Protein nitrogen determinations have been carried out on some of the fractions obtained by centrifugation. The protein nitrogen content of 'large' and 'small' granule fractions, separated as shown in Table 4, was determined in three experiments. The starting material, the low-speed supernatant (1), contained 1·08–1·32 mg of protein nitrogen per ml. (mean: 1·20 mg/ml.); the large granule fraction (2) contained 34–40% of fraction (1) (mean: 37%); the small granule fraction (3) contained 22–29% (mean: 25%), and the final supernatant 41–45% (mean: 43%). With the final washings (5) (mean: 4%), the protein in the starting material was thus satisfactorily accounted for. The values for 'large' and 'small' granule fractions and the final supernatant are similar to those reported for mitochondria, microsomes and soluble fraction, respectively, of rat liver (Schneider & Hogeboom, 1951).

In six of the experiments reported in Table 7, the protein content of 'top' and 'bottom' fractions was also determined. In the top layer 22·8–30·3% of

the dry matter was protein (mean: 26·4%), in the bottom layer 22·8–35·5% (mean: 29·2%).

Since the data for both pressor amine and protein were available from several experiments, we were able to calculate the adrenaline : protein ratio. These figures, which are given in Table 8, show that the large granule fraction had an adrenaline : protein ratio twice that of the low-speed supernatant from which it was derived. In all experiments in which top and bottom fractions were examined separately, the adrenaline : protein ratio was higher in the bottom layer; the mean figures were 0·22 for the top layer and 0·48 for the bottom layer.

TABLE 8. Adrenaline: protein ratio of fractions derived from bovine medullary homogenates

(The ratio has been calculated on a weight to weight basis. In Expt. no. 84 the centrifugation was as shown in Table 4; in this experiment the figures for adrenaline and protein are expressed in terms of mg per ml. of fraction. In the other experiments adrenaline and protein are expressed in terms of % of dry weight of fraction.)

Expt. no.	Fraction	Protein (mg/ml.)	Adrenaline (mg/ml.)	Adrenaline : protein ratio
84	Low-speed supernatant (1)	7·44	1·17	0·16
	Large granule fraction (2)	2·56	0·84	0·33
	Small granule fraction (3)	1·87	0·06	0·03
	Final supernatant (4)	3·12	0·20	0·06
	Final washings (5)	0·25	0·02	0·08
		%	%	
59	Top layer of sediment	23·2	6·8	0·29
	Bottom layer of sediment	22·8	16·0	0·70
61	Top layer of sediment	28·4	5·4	0·19
	Bottom layer of sediment	25·8	12·3	0·48
62	Top layer of sediment	22·8	5·2	0·23
	Bottom layer of sediment	34·4	17·0	0·49
87	Top layer of sediment	26·4	5·7	0·22
	Bottom layer of sediment	35·5	12·9	0·36
94	Top layer of sediment	30·3	5·2	0·17
	Bottom layer of sediment	30·5	11·8	0·39

Adrenaline : noradrenaline ratio in medullary fractions

In all experiments given in the preceding sections we determined the total pressor action of the various fractions against (−)-adrenaline as standard, but in a few experiments the adrenaline : noradrenaline ratio of the low-speed supernatant, the high-speed supernatant and the high-speed sediment was also determined. A preliminary account of these experiments, including a table of results, has already been given (Hagen, 1954*a*).

The method used in the adrenaline : noradrenaline determination was in all essentials similar to that described by Weil-Malherbe & Bone (1953); this is a fluorimetric method in which the adrenochrome (and noradrenochrome) is condensed with ethylene diamine to give a highly fluorescent product. When this method was used with homogenates of medullary fractions, the original material had to be highly diluted and an adsorption on alumina and subsequent

elution was found unnecessary. The final dilution of the material was made in 5 ml. of N-HCl; this was added directly to 1 ml. of ethylene diamine in a glass-stoppered test tube. The tubes were heated at 60–70° C for 30 min; the contents were then saturated with sodium chloride and the fluorescent products were extracted with *iso*butanol. In contrast to the finding of Weil-Malherbe & Bone (1953), the compound derived from noradrenaline, when tested with a blue filter, usually gave a more intense fluorescence than the corresponding compound derived from adrenaline; with the yellow filter the two compounds frequently had an almost equally intense fluorescence.

In one of the experiments already reported (Hagen, 1954*a*) the biological assay was not only carried out on the arterial blood pressure of the spinal cat, but the adrenaline : noradrenaline ratio was also determined by the method of Burn, Hutcheon & Parker (1950) on the normal nictitating membrane of the cat; the fluorimetric and biological methods agreed satisfactorily.

The data given (Hagen, 1954*a*) show that adrenaline and noradrenaline were similarly distributed between high-speed sediment and supernatant. The relatively high concentrations of noradrenaline found in the fractions are in agreement with some of the earlier data on bovine suprarenal glands; e.g. in the three medullae examined by Langemann (1951), using the method of Burn *et al.* (1950), the percentage of adrenaline was: 75, 50 and 50% respectively.

Effect of temperature on adrenaline retention

As long as the suspensions were kept at low temperature, little of the pressor amine content was lost from the granules. This is demonstrated by the results of those experiments of Table 1, in which the resuspended sediments were re-centrifuged. At higher temperatures, however, there was often a considerable release of adrenaline into the supernatant fluid. A number of incubation experiments, both at 25° and at 37·5° C, were carried out, in order to find out if the release of pressor activity could be affected by modifications of the conditions of incubation. These modifications included incubation either in oxygen or in nitrogen, addition of $MgCl_2$, of potassium phosphate, of ATP, of Versene (ethylene-diamine-tetraacetic acid), as well as of certain substrates of oxidation, such as α-ketoglutarate or glutamate. There was no indication that any of these modifications or additions had a significant effect on the release of pressor activity.

The action of acetylcholine and histamine

In preliminary experiments, no effect of the addition of either histamine or acetylcholine on the release of pressor amines was observed. In one experiment, 1·6 ml. samples of a resuspended high-speed sediment were incubated for 60 min at 37·5° C in O_2, one sample without, and another with, 0·5 mg of acetylcholine chloride; the suspensions were centrifuged and the pressor

activity of the supernatant fluids was determined. Both supernatant fluids had the same pressor activity.

The effect of histamine was tested in an experiment in which 3·5 ml. of resuspended sediment was left standing for 10 min at +2° C with and without 10 μg of the base. After centrifugation the supernatant fluids were tested: they had very much the same activities.

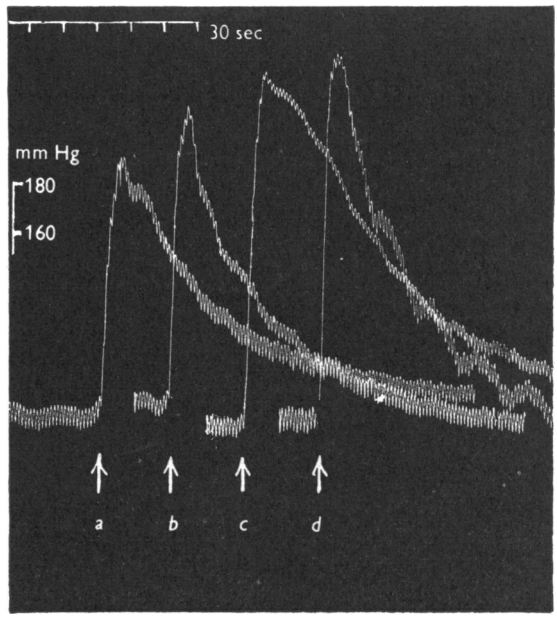


Fig. 2. Spinal cat. Arterial blood pressure. Injection of undenatured adrenal medullary granules. Intravenous injection at: (*a*) of 1·0 ml. of resuspended high-speed sediment in 0·25 M-sucrose corresponding to 4 mg of fresh tissue; (*b*) of 4 μg of adrenaline; (*c*) of 1·4 ml. of resuspended high-speed sediment in 0·25 M-sucrose; (*d*) of 6 μg of adrenaline.

Action of undenatured granules on the arterial blood pressure of the spinal cat

In the experiments described so far, adrenaline assays were always carried out after acidification and freezing, with subsequent thawing and neutralization. Experiments have also been done in which the freshly prepared fractions were injected intravenously, in order to determine whether the pressor amines were present in a fully active condition.

A tracing of the arterial blood pressure, recorded during such an experiment, is shown in Fig. 2. Two intravenous injections of (−)-adrenaline in 0·25 M-sucrose were made: at *b* 4 μg and at *d* 6 μg. At *a* 1·0 ml. of a 1 : 50 dilution in 0·25 M-sucrose of a resuspended high-speed sediment, as described in Table 1, was injected, and at *c* 1·4 ml. of the same dilution. It can be seen that the

ascending phase of the blood pressure rise after the injection of the suspended granules did not differ significantly from that after an injection of a solution of (−)-adrenaline. There was, however, a marked difference in the descending phase: the return of the blood pressure to normal after the injection of a resuspended sediment was much slower than after adrenaline.

The blood pressure rise at *c*, after the injection of 1·4 ml. of the granule fraction, had a maximum which was intermediate between that caused by 4 and 6 μg of adrenaline (*b* and *d*). However, the total adrenaline content of this granular fraction was not 5 μg but 22 μg; this latter value was derived from an acidified aliquot of the same fraction. In other words, only about 25% of the total amount of adrenaline injected at *c* had an immediate effect. The greater part of the pressor amine administered in this injection, about 75%, did not contribute to the immediate pressor rise; apparently, however, it was responsible for the prolonged descending phase of the blood-pressure curve.

TABLE 9. Maxima of blood-pressure response in terms of (−)-adrenaline of (*A*) acidified and (*B*) fresh fractions, suspended in 0·25 M-sucrose

Expt. no.	Type of fraction	*A* μg/ml. acidified	*B* μg/ml. fresh	*A*/*B*
1	Resuspended sediment (1st high-speed)	520	100	5·2
	Resuspended sediment (2nd high-speed	600	150	4·0
2	Resuspended sediment (2nd high-speed)	500	100	5·0
	Resuspended sediment (3rd high-speed)	400	60	6·7
	Supernatant (3rd high-speed)	30	33	0·9
3	Resuspended sediment (1st high-speed)	400	90	4·4
	Supernatant (1st high-speed)	140	180	0·8

Table 9 gives the result of a number of experiments in which freshly prepared granular fractions were injected intravenously into spinal cats; the total adrenaline content of acidified aliquots also is shown. In the granular fractions the adrenaline equivalents, as expressed in the blood-pressure maxima, varied from 15 to 25% of the total adrenaline content of the fraction. The immediate rise in blood pressure following the injection of the suspended granules cannot be attributed to adrenaline which had already diffused out of these particles. This is shown by Expt. 2 of Table 9, where recentrifugation of the suspension gave a supernatant fluid containing less than a third of the maximum immediate pressor activity of the original suspension.

The blood-pressure rise after the injection of undenatured supernatant fluid from high-speed centrifugation did not show a slow return to normal; the curves differed in no respect from those resulting from an injection of an aqueous solution of adrenaline. Table 9 (Expt. 3) shows that the blood-pressure rise after the injection of a fresh first high-speed supernatant fluid was not significantly different from the maximum obtained after the injection of an acidified sample of the same fraction. This shows that in the cytoplasmic sap

adrenaline is present in a form in which it is immediately available. A similar result was obtained when the supernatant after a third resuspension and subsequent high-speed centrifugation was examined (Expt. 2).

In the experiment shown in Fig. 2 and in those of Table 9 the fresh suspension was diluted with isotonic sucrose before injection. An entirely different result was obtained when distilled water replaced isotonic sucrose as the diluent

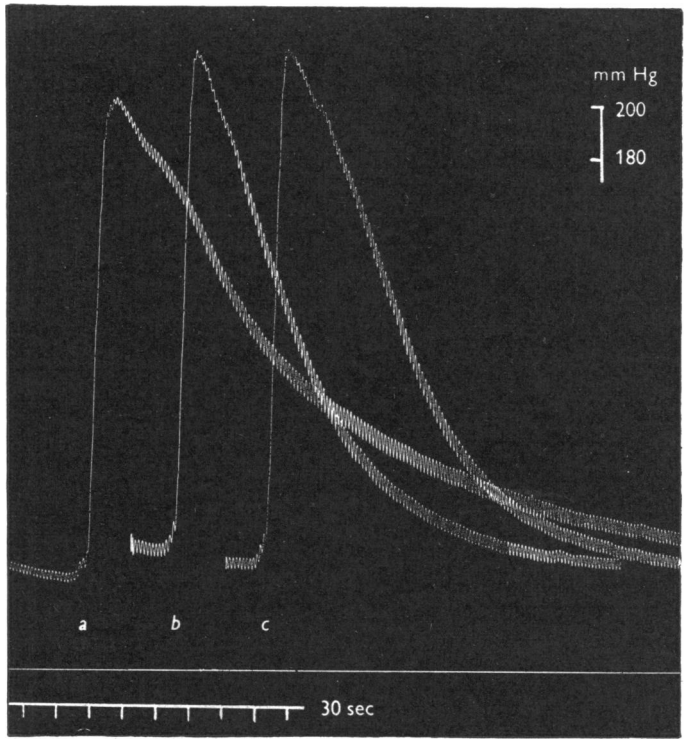


Fig. 3. Spinal cat. Arterial blood pressure. Comparison of the effect of adrenaline with that of suspensions of granules in isotonic sucrose or water. Intravenous injection at: (*a*) of 0·3 ml. of a sediment after a second high-speed centrifugation, resuspended in 0·25 M-sucrose (undenatured granules) corresponding to 30 mg of fresh tissue; (*b*) of 30 μg of adrenaline; (*c*) of 0·3 ml. of a 1 in 5 dilution of the same suspension in distilled water, corresponding to 6 mg of fresh tissue.

before injection. Fig. 3 is the blood-pressure tracing from an experiment in which at *a* 0·3 ml. of a suitable dilution of a resuspended sediment from a second high-speed centrifugation in 0·25 M-sucrose was injected intravenously. The subsequent injection, at *b*, was one of 30 μg (−)-adrenaline in 0·25 M-sucrose; at *c* 0·3 ml. of a 1 : 5 dilution, in distilled water, of the suspension injected at *a* was given. The tracing shows the slow return of the blood pressure to the original level after the injection of the undenatured fraction (at *a*). After treatment with distilled water (at *c*), an injection of one-fifth of the

amount of material given at *a* resulted in a blood-pressure maximum with a height similar to that given by the granules which had remained in 0·25 M-sucrose until administered; the return of the blood pressure to normal was prompt and comparable to that seen in *b*.

Whereas in the two experiments shown in Figs. 2 and 3 there was no indication that the blood-pressure rise after the injection of intact granules was slower, in the experiment shown in Fig. 4 the rise appeared to be a little retarded. Furthermore, in this experiment the slowing of the downward phase of the blood-pressure response was very marked. The second response, at *b*, was after the injection of one-fourth the amount of granules, but diluted in

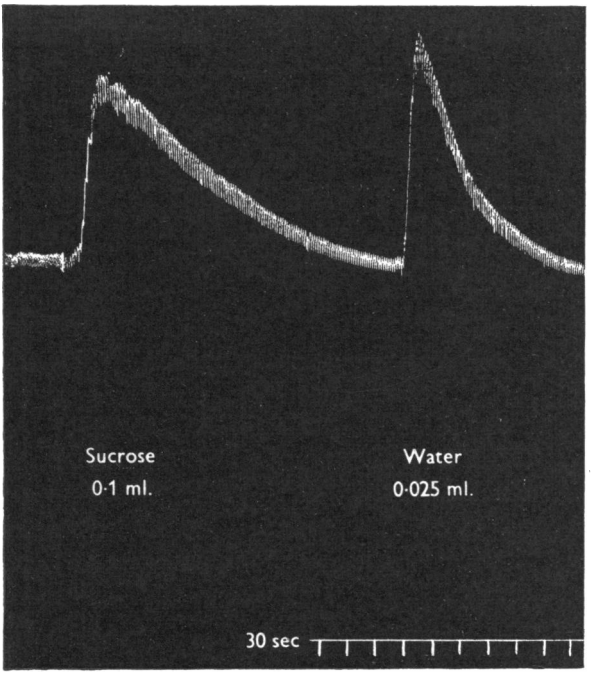


Fig. 4. Spinal cat. Arterial blood pressure. Intravenous injection: (*a*) of 0·1 ml. of a suspension of high-speed sediment in 0·32 m-sucrose, diluted to 1·0 ml. with 0·32 m-sucrose; (*b*) of 0·025 ml. of the same suspension, diluted to 1·0 ml. with distilled water.

the syringe, immediately prior to injection, with distilled water. Both rise and return of the blood pressure were fast, and the maximum attained was higher than with four times the amount of intact granules.

These observations show that, whereas in the first high-speed supernatant fluid, which contains the cytoplasmic sap, all the adrenaline was present in a form indistinguishable from an aqueous solution of adrenaline, this was not the case with the cytoplasmic granules. Here the adrenaline was present in a form in which it did not exert its full effect immediately. Furthermore, Figs. 3

and 4 show that after treatment with distilled water the total adrenaline was present in an immediately available form. The experiment described below shows that it was no longer present in the sediment.

A 1 in 10 homogenate of ox medulla in 0·32 m-sucrose was prepared; after an initial low-speed centrifugation at 950*g* for 30 min, a supernatant was obtained which was centrifuged for 20 min at 11,000*g*; the total volume was noted and the supernatant fluid from this centrifugation was discarded; the sediment was resuspended in one-quarter of the original volume of 0·32 m-sucrose. Two samples were prepared from this suspension; sample (*a*) contained 1·0 ml. of the suspension plus 3·0 ml. of 0·32 m-sucrose, and sample (*b*) contained 1·0 ml. of the suspension plus 3·0 ml. of water. The two samples were then again centrifuged for 20 min at 11,000*g*; the supernatant fluids were decanted and replaced by sucrose. Assay for pressor activity of acidified fractions showed the distribution:

Sample	(*a*)	(*b*)
Sucrose concentration	0·32 m	0·08 m
Supernatant	44 μg/ml.	580 μg/ml.
Resuspended sediment	508 μg/ml.	80 μg/ml.

In sample (*b*) practically all the activity was found in the supernatant fluid.

Localization of enzymes

Respiratory enzymes. As already briefly reported (Blaschko, 1954), the granular fractions, which contain pressor amines, also carry the bulk of the respiratory activity of the chromaffine cell. Evidence of the presence of the cytochrome-cytochrome oxidase system and of oxidation of the Krebs's cycle intermediates was obtained in manometric experiments.

The presence of the cytochrome system was demonstrated in experiments with *p*-phenylenediamine as substrate; in one experiment succinate was also used. From a homogenate of medullary tissue in 0·32 m-sucrose, a low-speed supernatant, a high-speed supernatant and a resuspended high-speed sediment were obtained as in the experiments described in Table 2. The three fractions were tested for 'indophenol oxidase' and 'succinoxidase activity'. Of each fraction, 3·0 ml. was incubated with 0·02 ml. of 3·13 % (w/v) $MgCl_2$ and 0·3 ml. of 10^{-2} M-substrate; the total volume was brought up to 4·0 ml. with water. The gas phase was oxygen; the incubation temperature was 25° C. Oxygen consumption in 20 min was:

	Oxygen consumption (μl.)		
		High-speed	
	Low-speed supernatant	Supernatant	Sediment (resuspended)
Without addition	45	0	0
With succinate	89	6	60
With *p*-phenylenediamine	97	13	66

This result indicates that the bulk of the succinoxidase and indophenol oxidase activity was present in the resuspended sediment from high-speed centrifugation.

In the experiment described in Table 5, in which the 'large granule' fraction and the 'small granule' fraction were collected separately, we have also determined the succinoxidase activity. The result of this experiment is shown in Table 10. It can be seen that most of the succinoxidase activity was present in the 'large granule' fraction, in agreement with what is known from other tissues.

TABLE 10. Succinoxidase activity of medullary granules and supernatant fluids

(Expt. 84, in which the centrifugation procedure was that shown in Table 4, was carried out on the same fractions as the adrenaline assays of Table 5. Each manometer flask contained: 1 ml. of fraction, 0·6 ml. of 0·067 M-sodium phosphate buffer of pH 7·4 and either 0·4 ml. water or 0·1 M-sodium succinate. In air at 37·5° C.)

		μl. O_2 consumed per hr		
		per ml. of fraction		
			% of (1)	per mg of protein
Low-speed supernatant	(1)	268	—	36
Large granule fraction	(2)	158	59	62
Small granule fraction	(3)	35	13	6
Final supernatant	(4)	12	5	4
Final washings	(5)	0	—	0

TABLE 11. Succinoxidase activity of 'top' and 'bottom' layers

(On these layers, adrenaline assays and protein determinations were also carried out (see Tables 7 and 8). Gas: air; temp. = 25° C. Each manometer flask contained 2·0 ml. of the suspended sediment, 0·68 ml. of 0·067 M-potassium phosphate buffer of pH 7·4, 0·02 ml. of 3·13% $MgCl_2$ and 0·3 ml. water or 0·1 M-sodium succinate.)

	'Top' layer, μl. O_2 consumed/hr			'Bottom' layer, μl. O_2 consumed/hr		
Expt. no.	per mg wet weight	per mg dry weight	per mg protein	per mg wet weight	per mg dry weight	per mg protein
61	1·85	10·3	36·3	1·75	6·9	26·7
62	2·28	12·3	54·0	2·40	8·9	25·8

In two of the experiments in which 'top' and 'bottom' layers were separately collected, we have also determined the succinoxidase activity. Results are shown in Table 11; the experiments are the same as Expt. no. 61 and Expt. no. 62 in Table 7. It can be seen that both fractions had good succinoxidase activity, but the oxygen consumption per unit of dry weight as well as per unit of protein was higher in the 'top' layer.

The oxidation of other Krebs's cycle intermediates, e.g. α-ketoglutarate or citrate, was less readily demonstrated. This is not surprising, as the preparations used were from slaughterhouse material. However, in a number of experiments both α-ketoglutarate and citrate were found to be oxidized.

Amine oxidase. In the rat liver, amine oxidase is known to be chiefly located in the large granule fraction (Cotzias & Dole, 1951; Hawkins, 1952). In the adrenal medulla, amine oxidase was found similarly distributed. In one experiment the 'large granule' fraction, the 'small granule' fraction and the final supernatant fluid were tested, using 0·1 M-tyramine hydrochloride as substrate; the oxygen consumed by 1 ml. of fraction, corresponding to 100 mg fresh tissue, during the first half hour in O_2 at 37·5° C was

Large granules	Small granules	Final supernatant
25·5 μl. O_2	7 μl. O_2	1·5 μl. O_2

DOPA decarboxylase. Langemann's (1951) finding that this enzyme occurs in the adrenal medulla has since been confirmed by Sourkes, Heneage & Trano (1952) and by Holtz & Bachmann (1952). The latter authors, working with hog adrenal medulla, found that in their extracts the apoenzyme was not always fully saturated with coenzyme, pyridoxal-5-phosphate (see also Holtz, Bachmann & Carsten, 1952). With our medullary fractions, we have also noted that the enzyme was not always maximally active, and we have therefore in the manometric experiments added to each flask 10 μg of pyridoxal-5-phosphate as the calcium salt. In order to reduce CO_2-retention the fractions (in sucrose) were brought to pH 6·5 by adding 0·067 M-sodium phosphate buffer.

In all these experiments the entire decarboxylase activity of the homogenates was present in the supernatant fluid after high-speed centrifugation; the sediment from high-speed centrifugation was entirely without decarboxylase activity.

Choline oxidase. In agreement with Langemann's finding (1951), no choline oxidase activity was detected in the medullary homogenates.

DISCUSSION

The observations described above confirm and extend our earlier finding that most of the pressor amines in sucrose homogenates of the adrenal medulla are present in a granular fraction. Several reasons are in favour of assuming that the distribution, as studied in the sucrose homogenates prepared at low temperature, is representative of that in the chromaffine cell. In 1940, Feldberg prepared saline homogenates of medullary tissue, presumably at room temperature; he found only a fraction of the total pressor activity in the sediment after centrifugation. We have noticed sometimes that in saline media (in NaCl or in KCl) release of considerable amounts of adrenaline had occurred. Raising the temperature also favoured release. It seems, therefore, that the granules of the chromaffine cells, as other intracellular granules, are preserved at low temperatures in non-electrolyte media. Moreover, the observations on

sheep medullae suggest that post-mortem changes occur which set free pressor amine from the granules.

That the adrenaline in the chromaffine cell is present in the form of granules, was suggested by several histologists (see Cramer, 1918*a*, *b*). The milder methods used in the present work have made it possible to isolate from the cells granules with a high adrenaline content. It is of interest to note that in Feldberg's (1940) experiments the small amount of adrenaline present in the 'debris' was retained upon re-centrifugation, but that it was released into the supernatant fluid upon addition of lysolecithin. A release of adrenaline from granular material by lysolecithin has recently also been described by Hillarp & Nilson (1954); these authors have also observed the release of adrenaline in hypotonic media (Hillarp, Lagerstedt & Nilson, 1953). All these observations suggest that the granules possess a semi-permeable membrane which shares certain properties with the cell membrane. Our observation that in the granules most of the adrenaline is stored in a form in which it is not able immediately to exert its effect upon the arterial blood pressure is in conformity with a concept of the storage of the amines in a space surrounded by a membrane.

The observation that in the medullary granules adrenaline is stored in a structure in which it is prevented from exerting its full pressor effect and from which it is released in a hypotonic medium, is closely analogous to observations on another amine, histamine. Here again it can be shown that the amine is carried in a fraction of granules and that the injection of fresh granules is without action upon the arterial blood pressure, but that the histamine can be fully released by lowering the osmotic pressure of the suspending medium (Hagen, 1954*b*).

The findings reported in this paper extend our earlier observations (Blaschko & Welch, 1953) in that they show that the large granule fraction is not homogeneous, but that it contains a component with a very high pressor amine content. The quantitative data given must, for a number of reasons, be considered as approximations. Our assays were carried out against adrenaline as standard. The ox medulla contains also some noradrenaline which has, weight for weight, slightly more pressor activity. This is a factor which makes the assay figures appear a little too high. However, the granules were not entirely free of sucrose solution and possibly not of small granules, and these are factors which would lower the adrenaline concentration. We believe, therefore, that the reported measurements of adrenaline give a reasonably good approximation to the real pressor amine content of the fractions.

The question as to the identity of our fractions with mitochondria must remain open until a full histochemical investigation of the granules has been carried out. The sedimentation properties of our 'large' granule fractions are similar to those described for liver mitochondria. That the mitochondrial fraction of liver cells is not homogeneous, but that there exist 'large' granules

with differing properties, has been described by a number of observers (Novikoff, Podber, Ryan & Noe, 1953; de Duve, Gianetto, Appelmans & Wattiaux, 1953). In the medullary homogenates, we have shown that the large granule fraction can be subdivided into two components with differing properties. Again the question might be asked: is this separation into 'top' and 'bottom' fractions an artifact which arises in the course of the disintegration of the chromaffine cells and the subsequent separation of the fractions? It is quite conceivable that the granules are components of larger organelles of the living cell, but it is difficult to see how the process of separation could have brought about a redistribution which would have led to an increase in the pressor amine content of one fraction at the expense of another. Accordingly, we feel justified in assuming that in the chromaffine cell there exists a structure with a pressor amine content of 12–17% of its dry weight. This high pressor amine content also makes it likely that we are now approaching homogeneity: if we interpret the relatively high succinoxidase content of the 'bottom' fraction as due to the admixture in the fraction of a granule high in succinoxidase activity, but free of amines, this would mean that the amine content of the granules in the fraction which was rich in amines had an even higher concentration of amines than the fraction as a whole. There must clearly be an upper limit to the possible adrenaline concentration of any one granule.

The adrenaline assays have shown the presence in the chromaffine cell of an organelle in which the concentration of pressor base alone is higher than the total concentration of base in extracellular fluids. At the pH of the tissue fluids, adrenaline can be considered as fully dissociated. It would therefore contribute fully to the osmotic pressure if present in aqueous solution. An osmotic disequilibrium between intracellular particles and the body fluids is now assumed to exist by many authors (see Bartley, Davies & Krebs, 1954); this disequilibrium is believed to be maintained at the expense of reactions that yield energy. The granular fractions which carry adrenaline have been shown to respire actively. However, the fact that the amines are well retained within the granules without oxygen consumption indicates that energy is not required for the maintenance of an osmotic disequilibrium. In order to account for our findings, we must therefore assume that the amines are present, at least in part, in a form in which they are osmotically inactive. In other words, they are believed to be present in a bound form.

At present, it is not possible to say in what form the adrenaline is bound. Three possibilities might be discussed: (1) the adrenaline might be present in a lipoid phase; (2) it might be held by a chelating agent, e.g. a metal; (3) the adrenaline ion might be held by negatively charged groups in the tissue constituents, e.g. by carboxyl, phosphate or sulphate groups. It is possible that more than one type of binding occurs. The ease and rapidity, however, with which the adrenaline can be detached in a fully active form makes the first of

these possibilities unlikely. The answer to these questions must be left to further work.

Closely linked with the mode of binding of the adrenaline is that of its release. Two possibilities have to be considered: either the granules are secreted as such and disintegrate only after they have left the chromaffine cell or the adrenaline is released within the cell into the sap and is then secreted across the cell membrane. There are observations which can be used in support of either possibility. Cramer (1918*a*) reported having seen granules in the venous sinus of the medulla. We have shown that fresh granules, injected intravenously, do exert an immediate effect upon the arterial blood pressure of the spinal cat; this effect, however, is only a small part of that of their total pressor amine content. The sustained pressor effect shows that some at least of the adrenaline, not immediately available, also exerts an effect. Accordingly, the possibility that the granules are secreted by the cell cannot altogether be ruled out. However, the presence of adrenaline in the supernatant fluids makes it likely that some adrenaline is present in the cytoplasmic sap; it seems quite possible that this is the amine that is secreted when the cell is stimulated.

The fact that some of the adrenaline in the fresh granular fractions is immediately available while most of it is not, can be interpreted in various ways. Some of the adrenaline might be present outside the granule, while the bulk is protected by a membrane. Or it is possible that granules exist which have a different degree of lability in the circulation of the cat: some which disintegrate immediately and others which remain intact somewhat longer. Or the adrenaline immediately available is the adrenaline present in free aqueous solution; the bulk of the adrenaline which does not contribute to the immediate pressure rise, represents the bound adrenaline.

An understanding of the mode of release will depend upon whether the adrenaline leaves the cell in granules or in aqueous solution. If the intact granules leave the cell, the main effect of stimulation would be upon the cell membrane. If the adrenaline leaves the cell across the cytoplasmic sap, we would have to postulate an intracellular mechanism which causes a redistribution of pressor amines within the chromaffine cell. The failure of substances like acetylcholine and histamine to release adrenaline from the granules could easily be understood on the basis of either of these two modes of release. The action of releasing agents, however, remains to be more thoroughly studied.

The sequence of chemical reactions by which adrenaline and noradrenaline are elaborated is not yet known. The work of Langemann (1951) has shown that in chromaffine tissue there occurs a very active enzyme which forms dopamine, an amine with sympathomimetic action. In the sucrose homogenates this enzyme is found in the supernatant fluid; the granular fractions were without enzymic activity. This suggests that in the living cell the decarboxylase is present in the cytoplasmic sap. If we provisionally assume that DOPA

decarboxylase is so located in the living cell and that it is in fact important in adrenaline and noradrenaline synthesis, it follows that amine formation occurs in the cell sap, whereas adrenaline and noradrenaline are chiefly present in the granules. We would then have to assume movement of amines in the cell in two directions: movement of newly made amine from the cytoplasmic sap to the granules and movement of adrenaline and noradrenaline stored in the granules into the cell sap. The further chemical changes which lead to adrenaline and noradrenaline may occur in the granules; this has already been discussed elsewhere (Blaschko, 1954). This question can only be answered by new experiments.

SUMMARY

1. Homogenates of bovine suprarenal medulla in cold isotonic or hypertonic sucrose contain about three-quarters of their pressor activity in a fraction which can be spun down at 11,000*g*.

2. The pressor amines are contained in a granule fraction with sedimentation properties similar to those of mitochondria.

3. A layer of granules has been separated with a pressor activity corresponding to an adrenaline concentration of 11·8–17·0% of the dry weight.

4. Intravenous injection of undenatured granules suspended in isotonic media causes a blood-pressure rise in the spinal cat which indicates that only a small part of the adrenaline is immediately active; granules suspended in distilled water cause an immediate blood-pressure rise which corresponds to their total pressor amine content.

5. The granular fractions of the chromaffine tissue also contain the respiratory enzymes of the cells and the amine oxidase activity.

6. DOPA-decarboxylase activity was found in the supernatant fluid after high-speed centrifugation.

We are grateful to have been able to pursue these investigations in the laboratory of Prof. J. H. Burn during the tenure by one of us (A.D.W.) of a Senior Fulbright Fellowship and by another (P.H.) of a C. J. Martin Fellowship of the National Health and Medical Research Council of Australia. We are grateful to Miss Jean M. Himms for skilful assistance. A few of the experiments were carried out during a stay of one of us (H.B.) at the Department of Pharmacology, Yale University Medical School; the help of the Lilly Research Foundation, which made this stay possible, is gratefully acknowledged. The work at Yale was supported by a grant from the National Institutes of Health.

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