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THE METHYLATION OF *NOR*ADRENALINE BY MINCED SUPRARENAL TISSUE

BY

EDITH BÜLBRING

From the Department of Pharmacology, Oxford

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It has now been established that the pressor activity in extracts from the suprarenal glands is not due to the presence of adrenaline only but also to the presence of *nor*adrenaline. This has been shown for the pig's gland by Schümann (1948, 1949) and for the dog's gland by Bülbring and Burn (1949a) by biological estimation. That both substances are present in extracts of suprarenal glands from cattle was shown by v. Euler and Hamberg (1949) who compared the results obtained by biological methods with those obtained by paper chromatography (James, 1948) which they modified to yield quantitative results. Goldenberg, Faber, Aston, and Chargaff (1949) have also applied chemical and biological methods to show the presence of *nor*adrenaline in commercial extracts of adrenal medulla from cattle and found that these contained 12–18 per cent *nor*adrenaline; one sample contained as much as 36 per cent. Tullar (1949) has isolated *l-nor*adrenaline from such extracts.

The presence in the gland of the non-methylated compound may have two purposes. It may be an end-product which can be released into the blood-stream; it may also be the precursor for the synthesis of adrenaline by the gland.

There is recent evidence for the release of *nor*adrenaline itself from the suprarenal medulla as was first suggested by Meier and Bein (1948). According to Holtz and Schümann (1949) the suprarenal gland releases a substance which is not adrenaline and is most probably *nor*adrenaline during the pressor reflex following clamping of the carotid arteries. Bülbring and Burn (1949b) showed that in the eviscerated cat stimulation of the splanchnic nerve to the suprarenal caused release of a mixture of adrenaline and *nor*adrenaline; this was shown by the fact that the pressor effect of splanchnic stimulation could be matched by an infusion of adrenaline, but the ratio of contractions of the two nictitating membranes, one denervated and one normal, could only be matched by infusing a mixture of adrenaline and *nor*adrenaline. In order to see whether the admixture of *nor*adrenaline was due to deficient methylation a number of cats were fed with methionine. However, in these cats also splanchnic stimulation produced a release of *nor*adrenaline with the adrenaline. The only difference observed was that the gradual decline in the proportion of adrenaline secreted during repeated splanchnic stimulation seemed to be absent in the animals fed on methionine.

This result suggested that the suprarenal gland, though normally releasing *nor*adrenaline to exert a function of its own, was also capable of methylating *nor*adrenaline in order to increase its store of adrenaline. The likelihood of this mechanism had been put forward by Blaschko (1939, 1942).

The present paper is concerned with the conversion of *nor*adrenaline to adrenaline by minced suprarenal glands *in vitro*.

METHOD

Dogs and cats were used. The suprarenal glands were obtained by three different procedures. The first was to anaesthetize the dog quickly with ether, bleed it and remove the gland with the shortest possible delay. The second was to anaesthetize the dog with ether, open the abdomen and remove the glands by operation while the dog was kept alive. The third was used in cats. Under ether anaesthesia the cord was cut, the brain destroyed and artificial respiration was given. In some experiments the right splanchnic nerve was dissected by retroperitoneal approach and was cut. Then the left splanchnic nerve was similarly dissected and stimulated. In other experiments both splanchnic nerves were stimulated simultaneously. After 30 minutes' stimulation the cat was bled out and the glands were removed with the shortest possible delay.

The suprarenals were dried with filter paper and weighed. They were then transferred into an icecold mortar, cut with scissors and ground with icecold saline, 1 c.c. per 0.05–0.1 g. gland. Of this suspension 2 c.c. were transferred to a test tube, 0.3 c.c. M/15 phosphate buffer was added, and in different experiments varying amounts of choline chloride, racemic *nor*adrenaline hydrochloride and adenosine triphosphate (ATP) were added. Each tube thus contained:

2.0 c.c. extract containing 0.1–0.2 g. gland.
0.3 c.c. M/15 phosphate buffer.
0.3–0.6 c.c. containing varying amounts of choline chloride.
0.3–0.6 c.c. containing varying amounts of *nor*adrenaline.
0.4 c.c. containing 8 mg. ATP.
3.3–3.9 c.c. total volume.

The weight of choline chloride added was always the same as that of racemic *nor*adrenaline hydrochloride, i.e., twice the amount of *l-nor*adrenaline. In those experiments in which no *nor*adrenaline was added the amount of choline added will be specifically stated. If any of the ingredients was omitted the corresponding volume of saline was added instead. For each experiment one or several control samples were prepared: the final procedure adopted was to add 0.3 c.c. buffer and 1 c.c. *N* HCl to 2 c.c. extract; this was quickly heated to boiling and immediately cooled. The same ingredients were added to this boiled control sample as to the one with which it was to be compared; the two were incubated alongside. The usual incubation time was 1 hour. After incubation 1 c.c. *N* HCl was added to each sample, though not to the control sample if acid had been added before incubation. The sample was then transferred to an Erlenmeyer flask. One washing with 2 c.c. buffer and two washings with 2 c.c. saline were also transferred into the Erlenmeyer flask. Each acidified sample was quickly heated to boiling point, cooled and made up to such a volume that 0.1 g. gland was present in 10 c.c. This solution was used for the assays.

In some experiments samples were incubated in Thunberg tubes evacuated and filled with nitrogen. The assay was carried out by the following methods.

The rat uterus method (de Jalon, Bayo, and de Jalon, 1945). This assay is based on the antagonism between adrenaline and acetylcholine. Constant contractions are evoked by constant doses of acetylcholine at constant intervals. The activity of the samples is estimated by the reduction in the size of contraction compared with that produced by a standard adrenaline solution. The preparation is almost invariably 100 times less sensitive to *nor*adrenaline than to adrenaline. It has been discussed in detail in a recent paper by Gaddum, Peart, and Vogt (1949) and I have used the same procedure. I am indebted to Professor Gaddum for the suggestion of using an apparatus according to Schild (1947) with a solution of carbachol instead of acetylcholine. In the later part of the experiments a simplified automatic apparatus has been used. I am indebted to Mr. O. B. Saxby for its construction.

The *frog heart*, in which the ratio N/A of activity of *nor*adrenaline to adrenaline is not as low as in the rat uterus, was used in one experiment only.

The *rabbit's duodenum* was used in 3 experiments. The ratio N/A for the rabbit's duodenum varied between 1 and 2. Adrenaline was never found to be the stronger.

The *spinal cat's blood pressure* was used in all except two experiments. The ratio N/A was very variable. In 3 out of 15 experiments it was less than 1 and only in 2 cats was it 2. The range was from 0.615 to 2.

The *cat's nictitating membrane*. This method was used in 11 out of 15 experiments. It has been described in detail in a recent paper by Bülbring and Burn (1949b) and is based on the fact that the denervated nictitating membrane becomes relatively much more sensitive to *nor*adrenaline than the normal membrane which is more sensitive to adrenaline. Thus the ratio of the size of contraction by the denervated membrane to that by the normal membrane is greater the larger the proportion of *nor*adrenaline in the mixture. By this means it is possible to test the activity of an extract in a spinal cat not only by the effect on the blood pressure, comparing it with adrenaline, but also on the nictitating membranes by comparing it with an equipressor dose of a known mixture of adrenaline and *nor*adrenaline.

For the calculation of the results obtained by different methods of different sensitivities a formula has already been used by Professor J. H. Gaddum (personal communication). I am grateful to Mr. J. St. L. Philpot for his advice in developing a formula as follows. Adrenaline was used as a standard throughout. Thus in a preparation of high sensitivity to adrenaline and of low sensitivity to *nor*adrenaline (e.g., rat uterus and frog heart) let 1 μg. *nor*adrenaline be equivalent to "a" μg. adrenaline. In a preparation of similar sensitivity to both let 1 μg. *nor*adrenaline be equivalent to "b" μg. adrenaline. Let the total activity on the rat uterus or frog heart be "U" (expressed as μg. adrenaline) and let the total activity on the rabbit's intestine or cat's blood pressure be "C" (expressed as μg. adrenaline). Then

$$U = A + aN$$
$$C = A + bN$$

where A is μg. adrenaline and N is μg. *nor*adrenaline. From these two equations the following formulae are derived:

$$A = \frac{bU - aC}{b - a}$$
$$N = \frac{C - U}{b - a}$$

These formulae were used for the calculations, of which two examples will be given.

The amount of *nor*adrenaline will be given throughout in terms of *l-nor*adrenaline.

Example 1

The estimation by the rat uterus method gave the following results for "U" in μg. adrenaline per c.c.

	Sample I	Sample II
1st strip	2.55	6.3
2nd strip	2.27	4.0
Mean "U"	2.41	5.15

The estimation on the cat's blood pressure gave for
Sample I "C" = 27.2 μg. adrenaline per c.c.
Sample II "C" = 25.8 μg. adrenaline per c.c.

The ration of activity $\frac{\text{noradrenaline}}{\text{adrenaline}}$ was on the rat's uterus "a" = 0.01, on the cat's blood pressure "b" = 1.8. From these results the values for adrenaline and *nor*adrenaline were calculated.

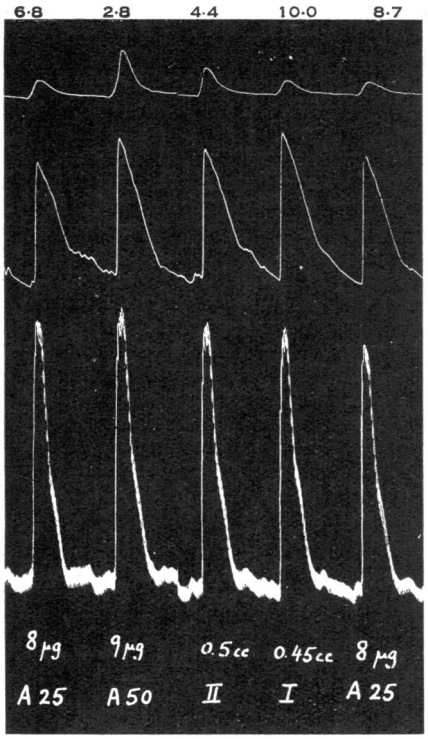

FIG. 1.—Assay of suprarenal extracts (Exp. 1) on spinal cat. Tracings are: arterial blood pressure (bottom), contractions of denervated (middle) and normal (top) nictitating membrane. Figures above are ratios of the size of contractions of the two membranes. I and II refer to samples in Exp. 1. "A" followed by a number indicates the percentage of adrenaline in a mixture of adrenaline and *nor*adrenaline.

$$A_{\text{I}} = \frac{1.8 \times 2.41 - 0.01 \times 27.2}{1.8 - 0.01}$$

$$= \frac{4.35 - 0.27}{1.79} = 2.27\ \mu\text{g. adrenaline}$$

$$N_{\text{I}} = \frac{27.2 - 2.41}{1.8 - 0.01} = 13.9\ \mu\text{g. } \textit{nor}\text{adrenaline}$$

$$A_{\text{II}} = \frac{1.8 \times 5.15 - 0.01 \times 25.8}{1.8 - 0.01}$$

$$= \frac{9.30 - 0.25}{1.79} = 5.08\ \mu\text{g. adrenaline}$$

$$N_{\text{II}} = \frac{25.8 - 5.15}{1.8 - 0.01} = 11.5\ \mu\text{g. } \textit{nor}\text{adrenaline}$$

Another estimate was obtained by recording the contractions of the cat's nictitating membranes and comparing the extracts with mixtures containing varying proportions of adrenaline and *nor*adrenaline. On top of the tracing shown in Fig. 1 are given the ratios of the size of contractions of the denervated to those of the normal membrane. The ratio for Sample II was between those for two mixtures containing 25 per cent and 50 per cent adrenaline respectively. The ratio for Sample I was less than that for the mixture containing 25 per cent adrenaline. The actual percentages in the extracts were obtained graphically from a curve relating the ratio of contractions of the nictitating membranes to the percentage adrenaline present in the standard solution injected (see Bülbring and Burn, 1949b). When this was done, as shown in Fig. 2, the percentage for those injections of extract, shown in Fig. 1, were 20 per cent adrenaline in Sample I and 37 per cent adrenaline in Sample II. Though the pressor effects in this stage of the experiment were gradually diminishing it may be seen that 0.5 c.c. sample II was $> 8\ \mu$g. mixture containing 25 per cent adrenaline and $< 9\ \mu$g. mixture containing 50 per cent adrenaline.

$\therefore$ 1 c.c. sample II $\equiv 17\ \mu$g. mixture containing >25 and <50 per cent adrenaline.

0.45 c.c. sample I $\equiv 8\ \mu$g. mixture containing 25 per cent adrenaline

$\therefore$ 1 c.c. sample I $\equiv 17.8\ \mu$g. mixture.

Estimations of this kind were repeated several times and the mean results were that

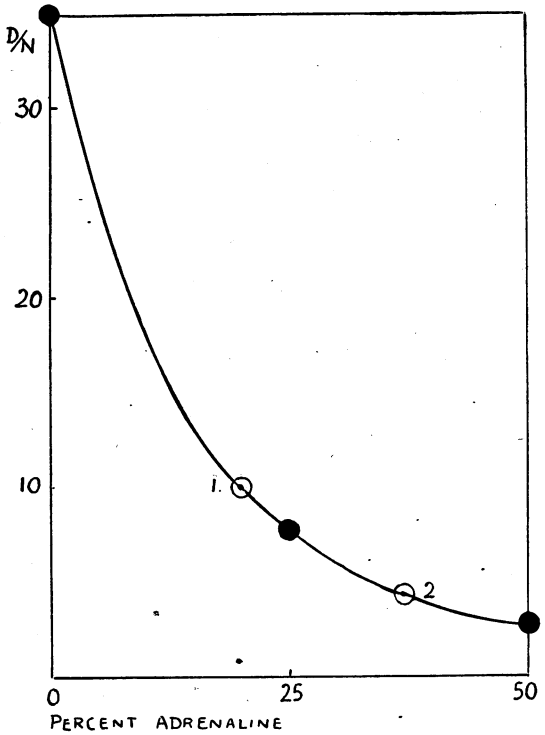

FIG. 2.—Curve relating ratio of the size of contractions of the denervated to the normal nictitating membrane (ordinates) and the percentage adrenaline in mixtures with *nor*adrenaline. The black circles represent effects obtained with standard solutions, white circles those obtained with suprarenal extracts.

Sample I contained 21 per cent adrenaline in 17.76 μg. total activity.

Sample II contained 34 per cent adrenaline in 17.5 μg. total activity.

As the samples represented a 1 in 100 dilution of the suprarenal tissue the activity could be calculated as μg. per g. gland. This is shown in Table I.

TABLE I

ACTIVITY IN SUPRARENAL EXTRACTS

Sample	Estimate	Preparation used for estimation	μg. per g. gland		
			Adrenaline	*Noradrenaline*	Total
I	1st	Rat uterus and cat blood pressure	227	1,390	1,617
	2nd	Cat nictitating membrane and blood pressure	372	1,404	1,776
		Mean	300	1,397	1,697
II	1st	Rat uterus and cat blood pressure	508	1,150	1,670
	2nd	Cat nictitating membrane and blood pressure	596	1,154	1,750
		Mean	552	1,152	1,704

The agreement of the results obtained by the different methods was very close. It was so in 10 out of 15 experiments. Estimations of total activity did not differ from the mean figure of several combined assays by more than 10 per cent in 14 out of 15 experiments. The deviation from the mean figure for the adrenaline content was more than 20 per cent in 4 out of 15 experiments. One of these will be taken as the second example.

Example 2 (This was the experiment in which the difference between the two estimates was greatest.)

The assay by the rat uterus method gave widely differing results for "*U*" in μg. adrenaline per c.c.

	Sample I	Sample II	Sample III
1st strip ...	2.25	4.8	2.05
2nd strip ...	2.9	4.85	2.4
3rd strip ...	5.65	9.8	4.25
Mean ...	3.6	6.5	2.9

The assay on the cat's blood pressure gave the following results for "*C*":

1 c.c. Sample I equivalent to 48 μg. adrenaline
1 c.c. Sample II ,, ,, 34 μg. ,,
1 c.c. Sample III ,, ,, 44 μg. ,,

With "*a*" = 0.01 and "*b*" = 2.0 the activity in μg. per g. gland was calculated and is shown as the first estimate for each sample in Table II. The much weaker pressor activity of Sample II than that of Sample I, when compared with adrenaline, is shown in Fig. 3.

TABLE II

ACTIVITY IN SUPRARENAL EXTRACTS

Sample	Estimate	Preparation used for estimation	μg. per g. gland		
			Adrenaline	*Noradrenaline*	Total
I	1st	Rat uterus and cat blood pressure	340	2,230	2,570
	2nd	Cat nictitating membrane and blood pressure	840	1,560	2,400
		Mean	590	1,895	2,485
II	1st	Rat uterus and cat blood pressure	640	1,400	2,040
	2nd	Cat nictitating membrane and blood pressure	1,170	1,130	2,300
		Mean	905	1,265	2,170
III	1st	Rat uterus and cat blood pressure	270	2,080	2,350
	2nd	Cat nictitating membrane and blood pressure	550	1,650	2,200
		Mean	410	1,865	2,275

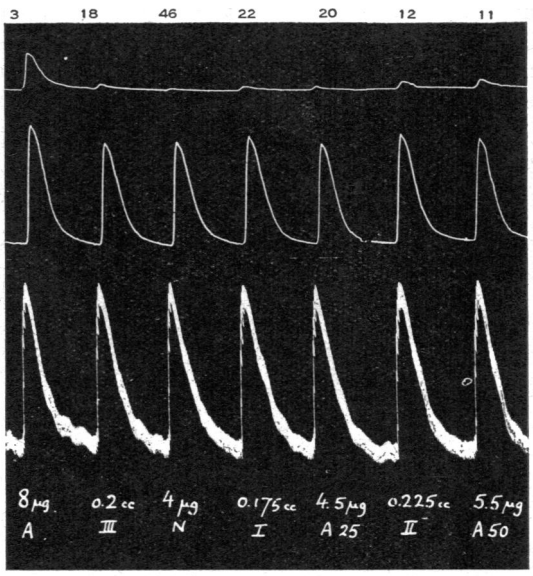

FIG. 3.—Assay of suprarenal extracts (Samples I, II, and III in Exp. 2) on spinal cat. Records as in Fig. 1.

8 μg. adrenaline = 4 μg. *nor*adrenaline
= 4.5 μg. mixture containing 25 per cent adr.
= 5.5 μg. mixture containing 50 per cent adr.
= 0.175 c.c. Sample I
= 0.225 c.c. Sample II
= 0.2 c.c. Sample III.

With these small doses, which were suitable for the assay of pressor activity, the normal nictitating membrane was scarcely affected. Larger doses had to be given for this purpose and part of the assay is illustrated in Fig. 4. By graphical determination from curves similar to that shown in Fig. 2 the proportion of adrenaline in the samples according to Figs. 3 and 4 were as follows:

	Sample I	Sample II	Sample III
Fig. 3	... 22 per cent	48 per cent	29 per cent
Fig. 4	... 38 ,, ,,	50 ,, ,,	16 ,, ,,

The mean figures of all estimations in this experiment are shown as the second estimates in Table II. The discrepancy between these and the other parallel assay is more than 100 per cent. However, the change in the three samples is in the same direction and of the same magnitude. This was so in every experiment without exception. No matter how large the absolute difference was between various assays for one sample (and it was never as large as in this sample), the relative difference between the samples was not affected; they always differed in the same direction. As there was no reason for assuming that either the assay combining results obtained on rat uterus and cat's blood pressure, or the assay in which extracts were compared with known mixtures on the nictitating membranes was more accurate than the other, the mean figure of both estimates was taken. The results were all converted to μg. per g. gland and are thus represented in the Tables.

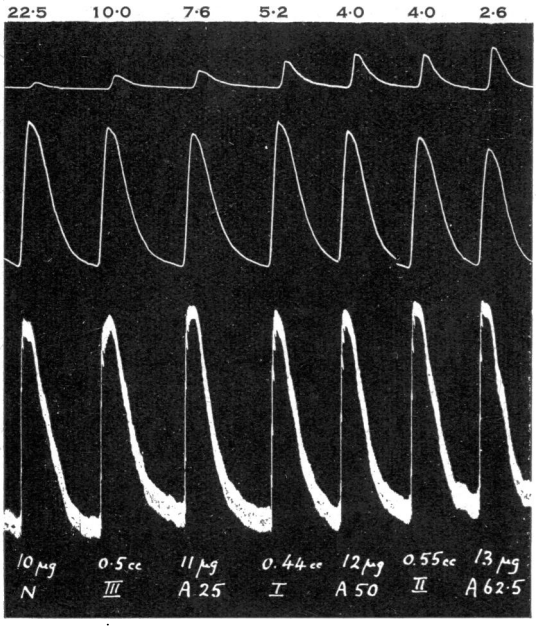

FIG. 4.—Assay (Exp. 2) continued. Records as in Fig. 1.

RESULTS

1. *The establishment of the control figure*

In order to observe a change in the proportion of adrenaline and *nor*adrenaline in a sample of suprarenal extract a control sample must be available which is subjected to the same conditions but in which enzyme activity has stopped. A comparison was therefore made on several occasions between extracts (*a*) acidified, boiled, incubated and not incubated, (*b*) non-acidified, boiled and then incubated, (*c*) the same, but with various additions. The results are given in Table III which shows the close agreement obtained by the combination of several biological methods. The total amount found in boiled control samples after one hour incubation did not differ by more than 6 per cent. from the samples which were not incubated. The agreement was close irrespective of whether they were acidified or not. A larger difference was found in Exp. 2 in the calculated total when a sample to which *nor*adrenaline had been added was compared with another without addition. Though the estimations of the amount of adrenaline and *nor*adrenaline in several samples of the same gland differed up to 20 per cent, the proportion of the two remained the same within 7 per cent. The largest differences were again found between estimations of samples to which *nor*adrenaline had been added and those without (Exps. 2 and 6), and also between samples incubated in O_2 and N_2 (Exps. 10 and 11). For this reason the control samples had the same additions and were subjected to the same conditions as those with which they were compared. The only exceptions are in experiments 4 and 5.

2. *The conversion of* nor*adrenaline to adrenaline*

Eight experiments were performed on dogs' suprarenals, the results of which are summarized in Table IV. The first three glands were removed by operation under ether anaesthesia, the other glands were removed as quickly as possible while the dog was bled out. In Exp. 6, in which the dog was used as blood donor for a heart-lung preparation, the delay before removal of the glands was considerable; this may be the reason for no change taking place during incubation. In all the other experiments a conversion of *nor*adrenaline to adrenaline was observed. This can only be assumed if together with the increase in adrenaline a corresponding decrease in *nor*adrenaline is found. Thus the values for

TABLE III

DOGS' SUPRARENALS. COMPARISON OF CONTROL SAMPLES

Amounts of adrenaline (adr.) and *nor*adrenaline (*nor.*) are given in μg. per g. gland

No. of experiment	Acidified, boiled, no additions. *Not* incubated		*Not* acidified, boiled, ATP added. Incubated in O_2		*Not* acidified, boiled, ATP and choline added. Incubated in O_2		*Not* acidified, boiled, ATP, choline and *nor*adrenaline added. Incubated in O_2		Acidified, boiled, ATP, choline and *nor*adrenaline added. Incubated in O_2		Acidified, boiled, ATP, choline and *nor*adrenaline added. Incubated in N_2		Deviation of estimations from mean
	μg.	%	μg.	%	μg.	%	μg.	%	μg.	%	μg.	%	%
Adr.			645	44	640	41	655	48					−1.2, +1.3
(2) *Nor.*			810	56	905	59	690*	52					−13, +13
Total			1,455		1,545		1,345						−7.5, +7.2
Adr.	550	23			580	22.5							±2.7
(3) *Nor.*	1,875	77			2,000	77.5							±3.2
Total	2,425				2,580								±3.1
Adr.	635	63.5			643	65	680	68					−2.8, +4.2
(6) *Nor.*	365	36.5			353	35	320*	32					−7.5, +5.5
Total	1,000				996		1,000						±0.2
Adr.							1,073	47	900	42	1,120	49	−15, +5
(10) *Nor.*							1,210	53	1,270	58	1,175	51	−3.5, +4.3
Total							2,283		2,170		2,295		−3.5, +2.1
Adr.							1,050	37			1,200	41	±7.3
(11) *Nor.*							1,770	63			1,700	59	±2.0
Total							2,820				2,900		±1.4

* The amount of *nor*adrenaline added has been subtracted in order to make the sample comparable with the other samples of the same gland

" Percentage change in adrenaline content " are calculated by taking into account not only the amount of adrenaline estimated but also the amount of *nor*adrenaline. In Exp. 1 an increase of 588 μg. adrenaline was found, but the decrease of *nor*-adrenaline was only 506 μg. The conversion is thus not more than 506 μg. or an increase of 99 per cent on the original amount of 511 μg. adrenaline.

The presence of ATP was found to be essential for the methylation of *nor*adrenaline. In four experiments samples incubated with ATP showed an increase of adrenaline, whereas parallel samples without ATP showed a decrease, which in Exp. 10 was accompanied by a corresponding increase in *nor*adrenaline, indicating the possibility of demethylation. The addition in one experiment (No. 2) of ATP alone caused no change in the proportion of adrenaline to *nor*adrenaline.

The amount of *nor*adrenaline present in the samples had an important influence though it did not wholly govern the extent of the reaction. Exp. 2 offers a good example. Sample *a*, to which only ATP was added, showed no change. Sample *b*, to which both ATP and choline were added, showed an increase in adrenaline which was similar to that of sample *c* to which *nor*adrenaline was added as well. None of the added *nor*adrenaline seemed to have been used. In two experiments (2*b* and 3*a*) in which the original gland was rich in *nor*adrenaline, a conversion to adrenaline was observed though only ATP and choline were added. But in two other experiments in which the gland contained originally very little *nor*adrenaline (5*a*) or none (4*a*) no increase in adrenaline was observed. In the latter experiment there is a further indication for a conversion in the opposite direction. When *nor*adrenaline was added to the samples as well (4*b* and 5*c*) some increase in adrenaline content was observed in both experiments, though surprisingly little considering the large amount added (2,000 μg. per g. gland). In Exp. 5*b* a sample incubated with ATP and *nor*adrenaline, but omitting the choline, nevertheless methylated a similar amount to that methylated in the one to which choline had been added.

There were thus four points emerging from Table IV: (1) ATP was essential for the conversion; (2) conversion was possible if ATP and choline were added under certain conditions; (3) these conditions depended to some extent on the amount of *nor*-

TABLE IV

DOGS' SUPRARENALS

Amounts of adrenaline and *nor*adrenaline before and after incubation in μg. per g. gland

No. of Exp.	Original			Final			Change			Percentage change in adr. content	Additions before incubation		
	Adr.	*Nor.*	Total	Adr.	*Nor.*	Total	Adr.	*Nor.*	Total		ATP	Ch	*Nor.*
1	511	912	1,423	1,099	406	1,505	+588	−506	+82	+99	+	+	833
2*a*†	645	810	1,455	615	745	1,360	−30	−65	−95	−5	+	−	−
b	640	905	1,545	1,110	350	1,460	+470	−555	−85	+73	+	+	−
c	655	1,940	2,595	1,100	1,570	2,670	+445	−370	+75	+57	+	+	1,250
3*a*‡	580	2,000	2,580	750	1,750	2,500	+170	−250	−80	+29	+	+	−
b‡				450	1,970	2,420	−130	−30	−160	−17	−	+	−
4*a*‡	1,340	0	1,340	1,130	225	1,355	−210	+225	+15	−15	+	+	−
b	1,340	2,000*	3,340	1,515	1,695	3,210	+175	−305	−130	+13	+	+	2,000
5*a*‡	1,048	212	1,260	1,040	224	1,264	−8	+12	+4	0	+	+	−
b	1,048	2,212*	3,260	1,308	1,820	3,128	+260	−392	−132	+25	+	−	2,000
c				1,358	1,816	3,174	+310	−396	−86	+30	+	+	2,000
d				845	2,120	2,965	−203	−92	−295	−10	−	+	2,000
6	680	1,320	2,000	695	1,385	2,080	+15	+65	+80	+2	+	+	1,000
10*a*	983	1,238	2,221	1,170	1,100	2,270	+187	−138	+49	+15	+	+	500
b				753	1,465	2,218	−230	+227	−3	−23	−	+	500
11*a*	1,050	1,770	2,820	1,290	1,385	2,675	+240	−385	−145	+23	+	+	1,000
b				1,047	1,664	2,711	−3	−106	−109	0	−	+	1,000

* Values are calculated, not estimated. † Choline chloride addition was 2.5 mg. per g. gland.
‡ Choline chloride addition was 4.0 mg. per g. gland.

TABLE V

CATS' SUPRARENALS

s = splanchnic nerve stimulated, o = no stimulation; adr., *nor.*, and total in μg. per g. gland

No. of Exp.	Original			Final			Change			Percentage change in adrenaline content	Additions before incubation		
	Adr.	*Nor.*	Total	Adr.	*Nor.*	Total	Adr.	*Nor.*	Total		ATP	Ch	*Nor.*
7*a* s	360	3,685	4,045	480	3,408	3,888	+120	−277	−157	+33	+	+	3,000
b o	452	3,353	3,805	630	3,225	3,855	+178	−118	+50	+26	+	+	3,000
8*a*†s	485	870	1,355	765	450	1,215	+280	−420	−140	+58	+	+	−
b†o	617	363	980	598	372	970	−19	+9	−10	−2	+	+	−
9**a*s	377	1,173	1,550	447	1,271	1,718	+70	+98	+168	0	+	+	540
b o	1,144	1,745	2,889	1,164	1,817	2,981	+20	+72	+ 92	0	+	+	620
12*a* s	1,000	3,260	4,260	1,790	2,500	4,290	+790	−760	+30	+76	+	+	1,500
b s				820	3,400	4,220	−180	+140	−40	−14	−	+	1,500
13*a* s	422	1,650	2,072	615	1,465	2,080	+193	−185	+8	+44	+	+	1,500
b s				422	1,545	1,967	0	−105	−105	0	−	+	1,500
14*a* s	300	1,397	1,697	552	1,152	1,704	+252	−245	+7	+82	+	+	1,500
b s				287	1,242	1,529	−13	−155	−168	0	−	+	1,500
15*a* s	590	1,895	2,485	905	1,265	2,170	+315	−630	−315	+53	+	+	1,500
b s				410	1,865	2,275	−180	−30	−210	0	−	+	1,500

* The samples in this experiment were incubated in N_2. † Choline chloride addition was 1 mg. per g. gland.

adrenaline present but the reaction was probably limited by the amount of enzyme available; (4) the addition of the three ingredients—ATP, choline, and *nor*adrenaline—caused a conversion in 6 out of 7 samples.

A surprising result was that glands removed under the most favourable conditions converted less *nor*adrenaline into adrenaline than those removed during a prolonged operation. As it is known that there is an increased splanchnic discharge during ether anaesthesia and laparatomy, the effect of splanchnic stimulation was investigated.

Cats were used for this purpose and the results are summarized in Table V. In the first three experiments the splanchnic nerve to one gland had been stimulated but not to the other; both glands were incubated under the same conditions with the addition of ATP, choline, and varying amounts of *nor*adrenaline. Of the latter an excessively large amount was added in Exp. 7 and a conversion was observed in both glands, which was slightly more on the stimulated side. In Exp. 8 no *nor*adrenaline was added before incubation and a large difference was seen between the activity of the stimulated as compared with the non-stimulated side. Though the total activity of the stimulated gland was not less than that of the non-stimulated side (in fact it was 37 per cent higher) the proportion of adrenaline was much less. When both suprarenal extracts were incubated with ATP and choline the proportion on the stimulated side became the same as that of the other side in which it remained unchanged. This is shown in Table VI. In Exp. 9 (Table V) no

TABLE VI

SUPRARENAL EXTRACTS INCUBATED WITH ATP AND CHOLINE

Gland	Original Adr.: *nor*adr.	Final Adr.: *nor*adr.
Stimulated ...	36 : 64	63 : 37
Non-stimulated	63 : 37	62 : 38

change was observed on either side; though the amount of adrenaline was increased there was no corresponding decrease of *nor*adrenaline. The result is not clear because the total activity was found to be considerably more than that of the control samples. This was the only experiment in which the control samples were heated in a water-bath for 5 min. instead of bringing them to boiling point quickly. Also it was the only experiment, recorded in Table V, in which the samples were incubated in nitrogen. This point will be discussed later. On the other hand, it may have been that too small an amount of *nor*adrenaline was added for this gland.

In the last four experiments in Table V both splanchnic nerves were stimulated, both suprarenals were pooled and the extracts incubated with an amount of *nor*adrenaline which was expected to be well above the amount of adrenaline present in the gland after prolonged splanchnic stimulation. This expectation was fulfilled as in each experiment the amount of *nor*adrenaline was found to be 3–4 times that of adrenaline. In each experiment the increase in the amount of adrenaline after incubation was considerable, i.e., 76, 44, 82, and 53 per cent. However, in parallel samples to which no ATP had been added no such increase was observed. One of these samples (in Exp. 12) gave yet another indication of a conversion in the opposite direction.

The experiments on cats' suprarenals confirmed the observation that the presence of ATP is essential for the methylation of *nor*adrenaline. The glands in which the store of adrenaline had been depleted by previous splanchnic stimulation appeared to have a high enzyme activity.

It might be argued that any change in adrenaline should not be calculated as a percentage change of adrenaline initially present in the gland but rather of the *nor*adrenaline. This would, however, give misleading results as the initial amount of *nor*-adrenaline depended largely on the very variable amounts added to different samples. For example, in sample 2*b* (Table IV) 470 μg. adrenaline was formed which is a conversion of 52 per cent of the initial 905 μg. *nor*adrenaline, while in sample 2*c* (of the same gland) the very similar formation of 445 μg. adrenaline represents only 23 per cent of the initial 1940 μg. *nor*adrenaline which was as high as this because 1,250 μg. had been added. Secondly, though splanchnic stimulation depleted the store of adrenaline the absolute figures of the initial content are on the whole similar to those of unstimulated glands and the highest absolute increase of 760 μg. occurred in a stimulated gland containing initially as much as 1,000 μg. adrenaline per g. (Exp. 12*a*, Table V).

Assuming that through the prolonged operative procedure in the first three dog experiments there occurred a certain amount of splanchnic stimulation the results may be summarized as in Table VII. (Exps. 6 and 9 have been omitted from this table as the absence of any change in either direction could probably be accounted for by faulty experimental procedure discussed above.) In glands depleted by splanchnic stimulation the increase in adrenaline content was larger than the variation found in control samples (see Table III). In 7 out of 10 samples it was more than 50 per cent and the mean

TABLE VII

EFFECT OF SPLANCHNIC STIMULATION

Stimulated glands			Non-stimulated glands		
No. of exp.	Percentage change in adrenaline content	Percentage change in total activity	No. of exp.	Percentage change in adrenaline content	Percentage change in total activity
DOGS					
1	+99	+ 5.8	4*b*	+13	−3.1
2*b*	+73	− 5.5	5*b*	+25	−3.2
2*c*	+57	+ 2.9	5*c*	+30	−2.6
3*a*	+29	− 3.1			
			10*a*	+15	+2.2
			11*a*	+23	−5.1
CATS					
7*a*	+33	− 3.9	7*b*	+26	−1.3
8*a*	+58	−10.4	8*b*	− 2	−1.2
12*a*	+76	+ 0.7			
13*a*	+44	+ 0.4			
14*a*	+82	+ 0.8			
15*a*	+53	−12.6			
Mean	+60.5		Mean	+18.6	

figure for all was an increase of 60.5 per cent from the original. On the other hand, in non-stimulated glands, no increase above 30 per cent was observed, only in 4 out of 8 experiments was it larger than the variation determined in control samples, and the mean figure for all was an increase of only 18.6 per cent. The variation in total activity was on an average no more than between several control samples. However, in two samples (Table VII; Exps. 8*a* and 15*a*) there was a loss of 10.4 and 12.6 per cent respectively. Nevertheless, in spite of this loss in total activity, the proportion of adrenaline was increased by 58 and 53 per cent respectively.

TABLE VIII

EFFECT OF ATP

No. of exp.	Suprarenal extracts incubated			
	With ATP		Without ATP	
	Percentage change in adrenaline content	Percentage change in total activity	Percentage change in adrenaline content	Percentage change in total activity
DOGS				
3	+29	− 3.1	−17	− 6.2
5	+30	− 2.6	−10	− 9.1
10	+15	+ 2.2	−23	− 0.1
11	+23	− 5.1	0	− 3.9
CATS				
12	+76	+ 0.7	−14	− 0.9
13	+44	+ 0.4	0	− 5.1
14	+82	+ 0.8	0	−10.0
15	+53	−12.6	0	− 8.5
Mean				
Dogs	+24.25		−12.5	
Cats	+63.75		− 3.5	

The experiments in which samples of the same gland were incubated with and without ATP are summarized in Table VIII. While there was always an increase of adrenaline when ATP was present there was either no change or a loss when ATP was absent. In Exp. 10, where the increase in adrenaline might not be significant it becomes more so when the loss of 23 per cent in the parallel sample containing no ATP is considered. On the average the loss in total activity does not exceed the variation in several control samples shown in Table III, but the samples containing no ATP were without exception found to be weaker than their control samples. The loss never exceeded 10 per cent.

3. *Experiments in aerobic and anaerobic conditions*

In four experiments a comparison was made between samples incubated in air and incubated in nitrogen. The results are shown in Table IX. It appears that methylation takes place anaerobically but not to the same extent as aerobically. Though the increase in the first two experiments is small and may not be significant, there is both in aerobic and anaerobic conditions a clear difference between samples incubated with and without ATP. The conclusion is drawn that anaerobic conditions are less favourable for the methylation of *nor*adrenaline and that this may be partly responsible for the negative result in Exp. 9 (Table V).

TABLE IX

EFFECT OF AEROBIC OR ANAEROBIC INCUBATION

No. of exp.	Percentage change in adrenaline content in suprarenal extracts incubated			
	In air		In nitrogen	
	With ATP	Without ATP	With ATP	Without ATP
10	+15	−23	+2	−22
11	+23	0	+17	−3
13	+44	0	+24	
14	+82	0	+58	

4. *The loss of total activity during prolonged incubation*

One experiment was carried out in which samples were incubated for different times. The loss was considerable, as shown in Table X. It may be seen

TABLE X

EFFECT OF DURATION OF INCUBATION

Dog's suprarenal, μg. per g. gland. Change during incubation

	Control		For 1 hour			For 1½ hours			For 2½ hours		
	%	μg.	%	μg.	Change	%	μg.	Change	%	μg.	Change
Adr. ...	22.5	580	30	750	+170	30	730	+150	33	690	+110
Nor. ...	77.5	2,000	70	1,750	−250	70	1,690	−310	67	1,410	−590
Total ...		2,580		2,500	−80		2,420	−160		2,100	−480

that nearly 20 per cent of the total activity was lost in 2½ hours. Though the proportion of adrenaline to *nor*adrenaline remained approximately the same up to 2½ hours the total loss of activity was then four times as much as the gain of adrenaline. This experiment indicates that the methylation of *nor*adrenaline proceeded quickly and was not limited by the time of incubation but by other factors.

Discussion

Blaschko's view (1939, 1942) that *nor*adrenaline is the precursor of adrenaline has been substantiated by the experimental results presented in this paper. *Nor*adrenaline and adrenaline are both present in suprarenal glands. They are both released, in varying proportions, from the gland during splanchnic stimulation, which might suggest that *nor*adrenaline is an end-product by itself. It has now been shown that it can be used by the suprarenal gland as precursor for the synthesis of adrenaline.

For the methylation of *nor*adrenaline by suprarenal extracts the presence of ATP appeared to be essential. No conversion took place in the absence of ATP. When *nor*adrenaline was added some samples converted a proportion of the added amount as well as the amount initially present in the gland. In other samples only a moderate degree of conversion took place in spite of a large surplus of *nor*adrenaline being available. Glands which had been subjected to prolonged splanchnic stimulation showed an increased capacity for the methylation of *nor*adrenaline, while glands removed as quickly as possible under the most favourable conditions converted relatively small amounts.

The conversion appeared to be completed within 1 hour (shorter periods have not been tested) during which the loss of total activity was negligible. If samples were, however, incubated for several hours a progressive loss in total activity was observed.

Holtz and Kroneberg (1948) proposed epinine rather than *nor*adrenaline as a precursor for adrenaline on the ground that *nor*adrenaline could be released from the suprarenal gland as an end-product. Recently (Holtz and Kroneberg, 1949) these authors have investigated the possibility of adrenaline synthesis by suprarenal extracts from phenylethylamine, tyramine, and oxytyramine. Incubation of extracts for several hours with the addition of any of these three amines produced an increase in pressor activity which the authors believe to be most probably due to the formation of adrenaline. It may, however, be due to various other reasons. Firstly, the control was neither acidified nor boiled. All the samples were shaken for several hours before the actual experiment was started in order to deplete the initial store. This depletion probably continued in the control samples during the period of the experiment while in samples to which the different amines were added the activity of amine oxidase might have been inhibited. Thus the stronger pressor activity could have been due not to a synthesis of adrenaline but to a prevention of its destruction.

On the other hand, a synthesis of adrenaline, either via epinine or via *nor*adrenaline, is considered by the authors. In the light of the experiments reported in this paper the synthesis of adrenaline is unlikely because no ATP was added. But the synthesis up to one stage before the end-product is possible. The synthesis of epinine is unlikely because of its feeble pressor activity. The synthesis of *nor*adrenaline is the most probable because it has a strong pressor activity and it may have been formed from oxytyramine during prolonged shaking in air. In their concluding remarks Holtz and Kroneberg (1949) correct their previous view by putting forward the theory that in the suprarenals the chief mechanism of adrenaline synthesis proceeds from oxytyramine via *nor*adrenaline to adrenaline. A side mechanism, which would enable the suprarenals to use phenylethylamine or tyramine for adrenaline synthesis, needs further analysis by suitable methods. Already in 1940 Vinet reported

experiments in which she incubated minced suprarenal tissue with hydroxytyramine and found a formation of what she believed to be adrenaline. For the estimation a colorimetric method was used, which, according to Euler does not distinguish between adrenaline and *nor*adrenaline. Thus in Vinet's experiments also a formation of *nor*adrenaline from hydroxytyramine might have taken place.

The methods employed for the assays in my experiments were far from accurate. It must, however, be emphasized that they agreed, without exception, in the estimation of the relative activity of different samples. The results are further strengthened by the use of known mixtures of adrenaline and *nor*adrenaline as standard solutions.

In a few samples the proportion of adrenaline to *nor*adrenaline was changed in the opposite direction indicating the possibility of the existence of a mechanism for the demethylation of adrenaline resulting in a formation of *nor*adrenaline. This has been suggested by Bacq and Fischer (1947) as one explanation for its presence in tissue extracts.

Summary

1. Suspensions of ground dogs' and cats' suprarenals are capable, during 1 hour's incubation at 37° C., of converting *nor*adrenaline to adrenaline.

2. The presence of ATP is essential for the methylation of *nor*adrenaline.

3. Glands removed after prolonged splanchnic stimulation have a higher methylating power than glands taken from a freshly killed animal.

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