Brit. J. Pharmacol. (1962), **19**, 355–364.

RETENTION OF INJECTED CATECHOL AMINES BY THE MOUSE

BY

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(*Received August* 3, 1962)

The importance of tissue storage as a method of inactivation of circulating catechol amines has been assessed by measuring the amount of unchanged hormone remaining in the mouse 30 min after the injection of various doses of [^{3}H]-adrenaline or [^{3}H]-noradrenaline. The results show that this method of inactivation is quantitatively more important for noradrenaline than for adrenaline at all dose levels studied, and that for both hormones storage is relatively more important at physiological dose levels (3 to 30 μg/kg) than at higher dose levels (150 to 300 μg/kg). The results obtained after the simultaneous injection of various doses of [^{3}H]-adrenaline and [^{14}C]-noradrenaline show that under certain conditions the two hormones compete for entry into the tissue storage sites. The possible nature of the mechanisms by which circulating catechol amines enter the tissue stores is discussed in the light of previous findings on the uptake of catechol amines by tissues *in vitro*.

Recent work has shown that the uptake and storage of circulating catechol amines by various animal tissues may represent an important mechanism in the physiological disposition of these hormones (Axelrod, Weil-Malherbe & Tomchick, 1959; Pennefather & Rand, 1960; Muscholl, 1960; Whitby, Axelrod & Weil-Malherbe, 1961; Strömblad & Nickerson, 1961). Comparison of the experiments of Axelrod *et al.* (1959) and Whitby *et al.* (1961) on the fate of tritiated adrenaline and noradrenaline after intravenous injection into mice indicated that this storage mechanism was quantitatively more important for noradrenaline than for adrenaline. Since the detailed data presented by these workers related to single injected doses of different amounts of adrenaline and noradrenaline, it was considered desirable to extend these experiments in order to determine whether the apparent difference between the uptake of circulating adrenaline and noradrenaline persisted over a wider dose range.

In the current studies the use of tritiated adrenaline and noradrenaline of high specific activity has allowed the administration of smaller doses than those originally employed. In addition, the use of combined doses of [^{3}H]-adrenaline and [^{14}C]-noradrenaline has made it possible to investigate simultaneously the fate of adrenaline and noradrenaline after the intravenous injection of a mixture of the two catechol amines, and to examine whether the storage mechanism of inactivation for each of the hormones is independent, or is affected by the simultaneous injection of the other hormone.

METHODS

Aluminium Oxide "WOELM" neutral, activity grade 1 was used after pretreatment as described by Whitby *et al.* (1961).

(±)-β-[^3H]-*Noradrenaline hydrochloride (20 mc/mg) and* (±)-β-[^3H]-*adrenaline hydrochloride (20 mc/mg),* obtained from New-England Nuclear Corporation, Boston, Mass., U.S.A., were supplied as solutions in dilute hydrochloric acid. Each material showed three major peaks of radioactivity after paper chromatography using n-butanol/acetic acid/water (4:1:1 v/v) as developing solvent. Pure radioactive adrenaline and noradrenaline were obtained by applying 1 ml. of each solution to sheets of Whatman no. 4 filter paper, developing the chromatograms in this solvent, and eluting with 0.01 N hydrochloric acid the strips corresponding in R_F to authentic adrenaline or noradrenaline (Axelrod, personal communication). Each eluate (3 ml.) was diluted with either non-radioactive (±)-adrenaline or (±)-noradrenaline to yield stock solutions containing 10 μg (free base)/ml. and 100 μc/ml. The stock solutions, which contained 2% (w/v) sodium metabisulphite, were stored at −15° C and remained chromatographically pure for at least seven months, as observed by Axelrod & Tomchick (1960).

(±)-2-[^{14}C]-*Noradrenaline acetate (130 μc/mg free base)* obtained from Nichem Inc., Bethesda, Md., U.S.A., was supplied chromatographically pure and was diluted to yield a stock solution containing 300 μg free base/ml. and 40 μc/ml. in 2% (w/v) sodium metabisulphite; this solution was stored at −15° C.

Doses for injection were prepared by further dilution of the stock solutions with water or non-radioactive (±)-adrenaline or (±)-noradrenaline so that each injected dose contained 1 μC [^{3}H]; the combined doses of [^{3}H]-adrenaline and [^{14}C]-noradrenaline were prepared similarly, to contain 1 μC [^{3}H] and 0.125 μc [^{14}C] unless otherwise stated. The volume of solution injected was 0.1 ml. in each case, and all data refer to doses of adrenaline and noradrenaline as μg free base.

Adult male albino mice (28 to 33 g) were weighed and then injected in the tail vein, killed by a blow on the head at various times after injection and homogenized in an MSE "Atomix" blender with an appropriate volume of cold 0.1 N hydrochloric acid to yield 200 ml. of homogenate in each case.

[^3H]-*Adrenaline,* [^3H]- *and* [^{14}C]-*noradrenaline* in samples of the homogenates were estimated by the method of Whitby *et al.* (1961); each analysis was performed in triplicate. Radioactive adrenaline or noradrenaline added to tissue homogenates and carried through the procedure was recovered in a yield of 60 to 70%, and the data were corrected for an average recovery of 65%.

Specificity of the procedure for measuring unchanged catechol amines: Homogenates of mice killed 30 min after the injection of 1 or 10 μg adrenaline and 1 or 45 μg noradrenaline were purified by the above procedure as far as the stage of elution from the aluminium oxide columns. The eluates were concentrated *in vacuo,* applied to Whatman no. 1 filter paper and examined by chromatography in three different solvent systems. The solvents were n-butanol:acetic acid:water (4:1:1 v/v); n-butanol saturated with 5% (w/v) aqueous trichloroacetic acid; and phenol:0.01 N hydrochloric acid (4:1 w/v) saturated with SO_2. The distribution of radioactivity on the papers was measured in a liquid scintillation spectrometer by the method of Wang & Jones (1959). In each case a single major peak corresponding in R_F to authentic adrenaline or noradrenaline accounted for 95 to 99% of the radioactivity on the chromatograms. The remaining 1 to 5% of the radioactivity was distributed in one or more minor peaks, none of which has been specifically identified.

Aliquots of the eluates from aluminium oxide columns were examined for the presence of non-amine catechols by the method of Kopin, Axelrod & Gordon (1961). This procedure would, for instance, have detected the presence of 3,4-dihydroxymandelic acid, but no more than trace amounts of these metabolites could be detected in any of the eluates.

[^3H]-Metanephrine and [^3H]-normetanephrine assay

[^{3}H]-Metanephrine and [^{3}H]-normetanephrine for use as reference materials were prepared enzymically from [^{3}H]-adrenaline and [^{3}H]-noradrenaline (Kopin *et al.*, 1961).

Free [^{3}H]-metanephrine and [^{3}H]-normetanephrine were assayed in mouse homogenates or urine by a method similar to that described by Kopin *et al.* (1961). Catechol amines were first removed by passing samples of the material through columns of aluminium oxide (Whitby *et al.*, 1961). The effluents from these columns were then adjusted to *p*H 6.5 and passed through columns of Amberlite CG-50 in the Na^+ form; these columns were washed with 20 ml. of water and the non-catechol amines were eluted with 10 ml. 4 N NH_4OH (Pisano, 1960). The eluates were concentrated to dryness *in vacuo* and taken up in 0.2 ml. water followed by 1 ml. methanol and then 3 ml. ethanol. The pooled extracts were centrifuged, and 3 ml. was added to 10 ml. phosphor for counting. Total [^{3}H]-metanephrine and [^{3}H]-normetanephrine were assayed by the same procedure in samples of homogenate or urine which had been previously incubated for 24 hr with "Glusulase" (Kopin *et al.*, 1961); conjugated [^{3}H]-metanephrine and [^{3}H]-normetanephrine were then determined by difference.

The recoveries of [^{3}H]-metanephrine and [^{3}H]-normetanephrine added to mouse homogenates and isolated by this method were 65 to 75% and the data were corrected for an average recovery of 70% (data from urine samples were corrected for an average recovery of 90%).

The specificity of this procedure was examined by applying samples of the concentrated eluates from the columns of Amberlite CG-50 to Whatman no. 1 filter paper and developing the chromatograms with n-butanol:acetic acid:water (4:1:1 v/v) or isopropanol:5% aqueous ammonia (4:1 v/v). In each case a single peak of radioactivity was found, corresponding in R_F to authentic metanephrine or normetanephrine.

Radioactivity measurements

All measurements were made in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 314a equipped with an automatic sample changer. The phosphor used was 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene in toluene. The method of Okita, Kabara, Richardson & LeRoy (1957) was used to determine [^{3}H] and [^{14}C] simultaneously. Internal standards were used to correct for quenching.

RESULTS

Relative amounts of unchanged catechol amine persisting in the whole mouse at various doses

Groups of mice were injected with various doses of adrenaline or noradrenaline; 30 min after injection the animals were killed and analysed for unchanged [^{3}H]-adrenaline or [^{3}H]-noradrenaline. Doses ranged from 0.1 μg to 50 μg noradrenaline and from 0.1 μg to 10 μg adrenaline. Doses of 100 μg noradrenaline or 25 μg adrenaline were lethal in most cases, and the number of animals successfully analysed at these doses was too few for presentation.

The results given in Table 1 show that between doses of 0.1 μg and 1 μg of each hormone there is no significant change in the relative amount of unchanged catechol amine remaining 30 min after injection. However, between doses of 1 μg and 5 μg adrenaline a significant fall ($P<0.001$) in the relative amount of unchanged adrenaline remaining is observed. Similarly between doses of 1 μg and 5 μg noradrenaline there is a significant fall ($P<0.001$) in the relative amount of unchanged noradrenaline remaining at the higher dose. The major part of each fall occurs in the dose range 1 to 2.5 μg catechol amine. At doses higher than 5 μg no further significant change in the relative amount of hormone remaining was noted for either adrenaline or noradrenaline.

TABLE 1

ANALYSIS OF UNCHANGED CATECHOL AMINE REMAINING IN THE WHOLE MOUSE 30 MIN AFTER THE INJECTION OF VARIOUS DOSES OF [^{3}H]-ADRENALINE OR [^{3}H]-NORADRENALINE

Data refer to mean values ± standard error of the mean. Numerals in parentheses indicate the number of animals used for each dose

Adrenaline or noradrenaline injected (μg)	% injected dose remaining as unchanged adrenaline	% injected dose remaining as unchanged noradrenaline
0·1	34·1±0·6 (5)	54·0±1·4 (6)
0·5	32·8±1·3 (5)	55·1±1·7 (6)
1·0	33·8±0·8 (8)	51·2±0·8 (7)
1·5	31·8±1·4 (8)	45·8±1·4 (5)
2·0	29·0±0·7 (6)	44·3±1·5 (6)
2·5	28·5±1·0 (13)	41·9±0·8 (6)
5·0	25·9±0·5 (6)	37·8±0·5 (6)
10·0	26·9±1·2 (5)	37·0±1·2 (6)
25·0	(Lethal dose)	35·5±2·3 (6)
50·0	(Lethal dose)	34·3±1·9 (6)

Simultaneous injection of adrenaline and noradrenaline

Groups of mice were injected with various combined doses containing [^{3}H]-adrenaline and [^{14}C]-noradrenaline. The amounts of unchanged [^{3}H]-adrenaline and [^{14}C]-noradrenaline remaining 30 min after injection were measured as before. The results of these experiments are given in Table 2.

The relative amount of adrenaline remaining 30 min after a single dose of 1 μg is 33.8% (Table 1). When 1 μg adrenaline is given together with 10 μg noradrenaline, the amount of adrenaline remaining 30 min after injection is 21.9%, a value significantly lower ($P<0.001$) than when 1 μg is injected by itself, but not significantly different from the value obtained after a single injection of 10 μg adrenaline (Table 1). The presence of 10 μg adrenaline has a similar effect on the disappearance of 1 μg noradrenaline when these doses are injected simultaneously. There was also

TABLE 2

ANALYSIS OF UNCHANGED ADRENALINE AND NORADRENALINE IN ANIMALS KILLED 30 MIN AFTER THE INJECTION OF COMBINED DOSES OF [^{3}H]-ADRENALINE AND [^{14}C]-NORADRENALINE

Data refer to mean values ± standard error of the mean. Numerals in brackets indicate number of animals at each dose. *P* values refer to a comparison of each individual dose with the same dose given singly (Table 1)

Injected dose (μg)	% injected adrenaline remaining	% injected noradrenaline remaining
0·5 Adrenaline+ 0·5 Noradrenaline	31·8±0·4 (7) ($P>0·05$)	50·1±0·8 (7) ($P<0·05$)
1 Adrenaline+ 1 Noradrenaline	29·4±1·6 (9) ($P>0·05$)	42·7±2·1 (9) ($P<0·01$)
2·5 Adrenaline+ 2·5 Noradrenaline	25·0±0·8 (6) ($P>0·05$)	41·2±1·0 (6) ($P>0·05$)
1 Adrenaline+ 10 Noradrenaline	21·9±1·2 (8) ($P<0·001$)	35·5±1·6 (8) ($P>0·05$)
10 Adrenaline+ 1 Noradrenaline	25·6±0·6 (6) ($P>0·05$)	37·8±0·6 (6) ($P<0·005$)

a significant reduction in the amount of noradrenaline remaining in mice 30 min after the injection of 1 μg of this hormone when given simultaneously with 1 μg adrenaline, as compared with the findings when 1 μg noradrenaline was injected by itself.

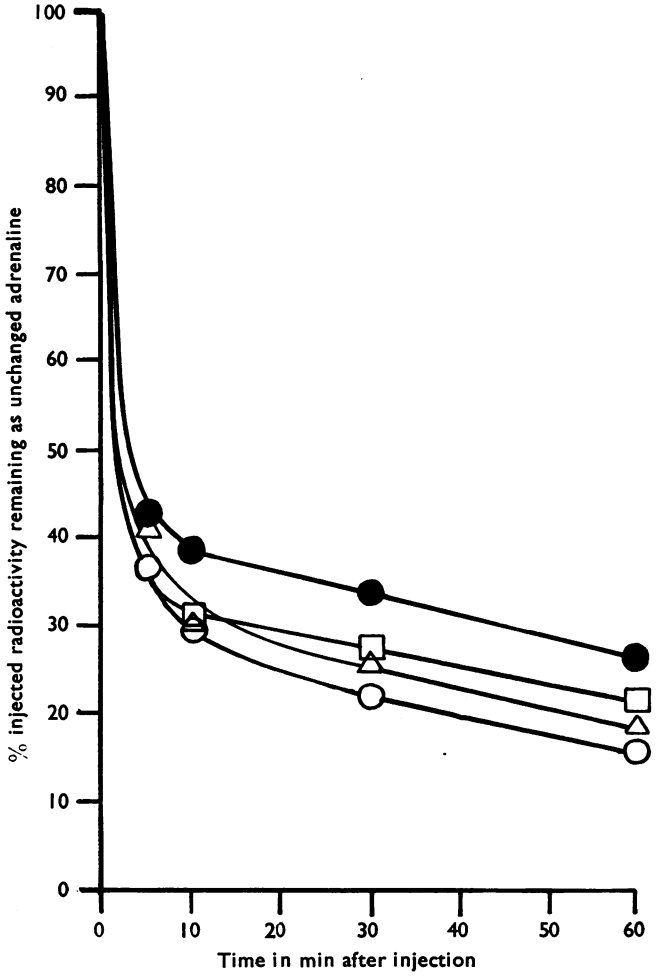

Fig. 1. The disappearance of adrenaline in the mouse after intravenous injection. Each point is the mean of from 3 to 8 animals. The injected doses were: ● 1 μg [^{3}H]-adrenaline, □ 10 μg [^{3}H]-adrenaline, ○ 1 μg [^{3}H]-adrenaline in the presence of 10 μg [^{14}C]-noradrenaline, and △ 10 μg [^{3}H]-adrenaline in the presence of 1 μg [^{14}C]-noradrenaline.

The time course of the disappearance of adrenaline and noradrenaline was followed after the injection of single doses of 1 μg or 10 μg of either hormone, and after the simultaneous injection of 1 μg of one hormone and 10 μg of the other. The results show (Fig. 1) that, when 10 μg noradrenaline is injected at the same time as 1 μg adrenaline, the amounts of adrenaline remaining in the animals at various times

after the injection are reduced, when compared with the amounts found after 1 μg adrenaline given singly. The simultaneous injection of 10 μg adrenaline has a similar effect upon the rate of disappearance of 1 μg noradrenaline (Fig. 2).

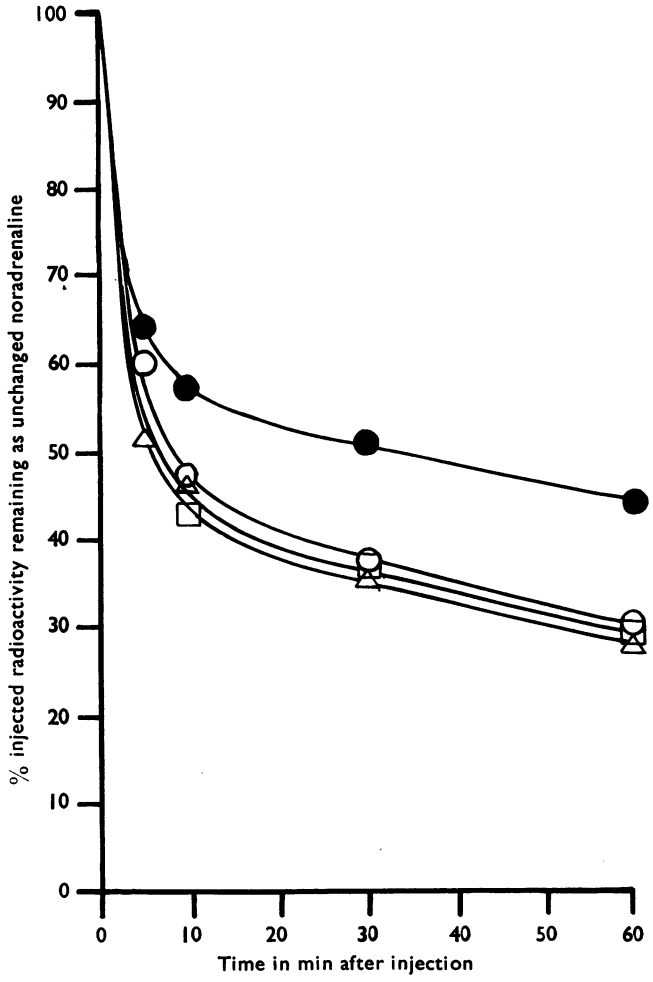

Fig. 2. The disappearance of noradrenaline in the mouse after intravenous injection. Each point is the mean of from 3 to 8 animals. The injected doses were: ● 1 μg [^{3}H]-noradrenaline, □ 10 μg [^{3}H]-noradrenaline, ○ 1 μg [^{14}C]-noradrenaline in the presence of 10 μg [^{3}H]-adrenaline and △ 10 μg [^{14}C]-noradrenaline in the presence of 1 μg [^{3}H]-adrenaline.

Analysis of O-methyl metabolites

The results of the analyses of animals killed 30 min after the injection of various doses of adrenaline and noradrenaline are given in Table 3. The figures represent the sum of metanephrine or normetanephrine found in the homogenates and in the urine excreted during the 30 min period; the amounts of excreted radioactivity,

TABLE 3

METANEPHRINE AND NORMETANEPHRINE ANALYSIS OF ANIMALS KILLED 30 MIN AFTER THE INJECTION OF A LOW OR A HIGH DOSE OF ADRENALINE OR NORADRENALINE

Injected dose (μg)	% injected dose present as free *O*-methylated metabolite	% injected dose present as conjugated *O*-methylated metabolite
1 Adrenaline	16·0	38·4
13 Adrenaline	20·0	38·3
1 Noradrenaline	19·0	26·5
30 Noradrenaline	27·5	23·0

however, contribute only a very small proportion to the total in each case. 85 to 95% of the injected radioactivity could be accounted for in terms of the total of unchanged hormone plus its corresponding 3-*O*-methylated metabolite (free and conjugated).

DISCUSSION

The results confirm the findings of Axelrod *et al.* (1959) and of Whitby *et al.* (1961) that, following an intravenous injection of adrenaline or noradrenaline into mice, these hormones are metabolized rapidly at first, but thereafter considerably more slowly. Significant amounts of unchanged tritiated hormone are detectable in the animals even after one hour. It has also been confirmed that *O*-methylation is the major route for the metabolism of circulating catecholamines ; 30 min after injection less than 15% of the administered radioactivity remains to be accounted for in terms of acidic or neutral metabolites, such as 3-methoxy, 4-hydroxymandelic acid.

The persistence of unchanged hormone in the animal has been interpreted (Axelrod *et al.*, 1959 ; Whitby *et al.*, 1961) as meaning that, following an intravenous injection or release of catechol amines into the circulation, a large percensage of the circulating hormone is removed from the circulation and inactivated by incorporation into tissue stores. It was further suggested that this bound, inactive catechol amine is thereafter slowly released and subsequently metabolized, thereby accounting for the slow continuing fall in the amount of unchanged hormone persisting in the animals. It has since been shown that tritiated noradrenaline in various tissues is located in the same store as endogenous noradrenaline—probably in or near post-ganglionic sympathetic nerve endings (Hertting & Axelrod, 1961 ; Potter & Axelrod, 1962 ; Hertting & Hess, 1962). The ability of certain tissues to accumulate circulating catechol amines has been demonstrated by other techniques. Muscholl (1960), Pennefather & Rand (1960), and Strömblad & Nickerson (1961) observed an increased noradrenaline content in various tissues after intravenous infusions of noradrenaline, and the latter workers reported a similar increase in the tissue content of adrenaline after intravenous infusions of adrenaline. The uptake of circulating catechol amines by tissues has also been inferred from pharmacological evidence relating to the ability of infused noradrenaline to restore the pressor response to tyramine in reserpinized animals (Burn & Rand, 1958), and to the ability of infused noradrenaline and adrenaline to restore vasoconstrictor effects in reserpinized animals (Rosell & Sedvall, 1961).

The findings of Axelrod *et al.* (1959) and of Whitby *et al.* (1961) have been considerably extended in the present investigation by studying the fate of a wide range of injected doses of adrenaline and noradrenaline. The results obtained show that the uptake of circulating catechol amines (as measured by the amount of unchanged hormone remaining in the animal 30 min after injection) is greater in the case of noradrenaline than with adrenaline, throughout the dose ranges tested.

Axelrod *et al.* (1959) stated that there was no difference in the rate of metabolism of adrenaline in the whole mouse for doses of from 1 to 10 μg. In the present experiments it has been found that there is a significant difference between the rate of disappearance of doses of 1 μg adrenaline or less and of doses of 5 μg or more. With the lower, more physiological doses (3 to 30 μg/kg) the rate of disappearance is slower than with the higher doses (150 to 300 μg/kg); at intermediate doses the rate of disappearance is between the two extremes, and there would seem to be no sharp line of demarcation of dosage below which 34% of the injected adrenaline remains after 30 min or above which only 26% remains. No explanation is offered for this discrepancy between the findings of Axelrod *et al.* (1959) and the results of the present series of experiments, although it is possible that it is due to the use of different strains of mice.

The effects of varying the dose of noradrenaline injected into mice are even more marked than in the case of adrenaline. A fall of 17% in the relative amount of unchanged hormone remaining 30 min after injection was noted between low and high dose levels, and there is again a gradual transition from the low-dose to the high-dose response over a relatively small part of the dose range, from 1 to 5 μg.

The demonstration that the uptake mechanism for each hormone is relatively more important at low-dose levels than at high, and the continued uptake of a smaller but constant proportion of the injected dose even at the higher dose-levels, suggests that the uptake of circulating catechol amines *in vivo* may be a process similar to that described for the uptake of tritiated catechol amines by tissue slices *in vitro* (Brodie, Dengler, Titus & Wilson, 1960; Dengler, Spiegel & Titus, 1961; Wilson, Murray & Titus, 1962). In these *in vitro* experiments the uptake of catechol-amines at very low levels in the medium was ascribed to a tissue concentrating mechanism which was able to produce a tissue concentration of tritiated catechol amine several times that of the medium. As the level of catechol amine in the medium was increased this mechanism became saturated, and further entry of catechol amine into the tissues was largely by simple diffusion, so that the tissue catechol amine concentration gradually approached that of the medium. A similar mechanism has been proposed for the uptake of serotonin and catechol amines by blood platelets *in vitro* (Hughes & Brodie, 1959). In terms of the current studies this would imply that the uptake of circulating catechol amines in the mouse is effected mainly by a tissue concentrating mechanism at low injected levels, but that this mechanism becomes saturated if the injected dose exceeds 1 μg (30 μg/kg) of either hormone. At doses greater than 1 μg further uptake is by passive diffusion and the overall process of uptake becomes quantitatively less important as a diminishing proportion of the whole uptake is due to the concentrating mechanism. Eventually, for doses of 5 μg or more, uptake is almost entirely by diffusion.

The results obtained with combined doses of adrenaline and noradrenaline suggest that a single concentrating mechanism may serve for the uptake of both hormones. Circulating noradrenaline and adrenaline at low concentrations compete for entry into the tissue storage sites by this route, so that a combined dose of the two hormones can saturate this uptake system even though the dose of either hormone would have failed to do so if given singly. On the other hand at higher blood concentrations the two hormones continue to enter the tissues by simple diffusion, which is not subject to any competition effects. In the present experiments it was possible to demonstrate significant competition in those cases where the combined dose was above the level required to saturate the concentrating mechanism, and where the dose of one hormone would have been below this saturating level had it been given singly. Competition could not be demonstrated for doses such as 2.5 μg of adrenaline + 2.5 μg noradrenaline, since the uptake of such doses even when given singly was largely by diffusion, each individual dose being sufficiently high to saturate the tissue concentrating mechanism. The failure to show significant competition effects with a simultaneous dose of 0.5 μg of each hormone was probably the result of using a combined dose which failed to saturate the concentrating mechanism. The present studies support the hypothesis that circulating adrenaline and noradrenaline compete for entry into tissue storage sites. This view has already been put forward by Strömblad & Nickerson (1961) on the basis of their findings that intravenous infusions of either hormone in the demedullated rat caused a lowering in the tissue content of the other hormone.

The results obtained here emphasize the importance of using experimental doses of catechol amines which are small enough to be comparable with those encountered under physiological conditions *in vivo*. The uptake and storage of circulating catechol amines after the administration of massive doses of these hormones may well have little physiological relevance.

It is a pleasure to acknowledge the financial assistance of a Scholarship (held by L. L. I.) and of a grant for materials and equipment (to L. G. W.) from the Medical Research Council.

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