Brit. J. Pharmacol. (1957), **12**, 306.

EFFECTS OF CHOLINE 2:6-XYLYL ETHER BROMIDE UPON THE SUPRARENAL MEDULLA OF THE RAT

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(RECEIVED MARCH 29, 1957)

The administration of choline 2:6-xylyl ether bromide (TM 10) daily to rats for two weeks depletes the suprarenals of about one-half their normal content of adrenaline and noradrenaline. This depletion has been demonstrated histologically and by colorimetric and biological estimation of the amines present in extracts of the glands. Treatment with TM 10 causes similar histological signs of depletion in autografts of adrenal chromaffin tissue in the rat iris. Restoration of catechol amines in rat suprarenals, previously depleted by TM 10, occurs slowly and appears to be complete 14 days after withdrawing the drug. These results, considered in conjunction with the estimated rate of turnover of catechol amines in the rat suprarenal, lend support to the view that TM 10 may interfere with the biosynthesis of these amines.

Choline 2:6-xylyl ether bromide (TM 10) displays several pharmacological properties, including amine oxidase-inhibitory, ganglion-stimulant, muscarine-like, and neuromuscular-blocking actions (Brown and Hey, 1956; Willey, 1957; Edge, Mason, and Wyllie, 1957). Many of its actions are weak or transient, but one which is both interesting and pronounced is the ability of the compound to block the activity of adrenergic nerves for prolonged periods (Hey and Willey, 1954). Exley (1957) suggested that TM 10 may interfere, in some way, with the biosynthesis of the adrenergic nerve transmitter. He showed that the drug, given systemically, reduced the amount of transmitter liberated on stimulation of adrenergic nerves, but did not seem to impair conduction of impulses along such nerves; moreover, cholinergic postganglionic nerves were not similarly paralysed by TM 10. Doses sufficient to paralyse adrenergic nerves did not, in acute experiments, influence the liberation of pressor amines from the stimulated suprarenals of cats.

In a further attempt to elucidate the mode of action of TM 10, the effects of the chronic administration of this substance have been observed upon the normal rat adrenal medulla, and upon adrenal medullary grafts, using both pharmacological and histological methods.

The results suggest that the daily administration of TM 10 causes a progressive depletion of pressor amines both from the normally innervated gland, and from the non-innervated graft, such as might be expected as a result of either an interference with the biosynthesis of catechol amines or a direct stimulant effect on the chromaffin cells themselves.

METHODS

Male albino rats of Sprague-Dawley strain were used. In all experiments animals of comparable age and weight were chosen; many were litter-mates. All the rats were maintained under identical conditions of nutrition and temperature.

In preliminary experiments rats were given convulsive doses of insulin (10 units soluble/100 g. body weight, s.c.) and, after each animal had suffered three convulsions, sufficient amounts of 5% glucose saline were given subcutaneously to allow complete recovery. The insulin-treated animals were then divided into two groups, the animals in one receiving daily injections of TM 10 (10 mg./kg., s.c.), and the animals in the other receiving injections of isotonic saline. The adrenals were compared histologically after five days of treatment.

In a second experiment rats were given daily subcutaneous injections of TM 10 (10 mg./kg.), or of choline phenyl ether bromide (0.1 mg./kg.), or of an equivalent volume of isotonic saline. Some animals were killed after 7 days, and the remainder after 14 days. The adrenal glands were examined histologically. TM 10 (10 mg./kg./day) was also administered to 2 rats which had adrenal medullary autografts in the anterior chamber of the right eye. The autografts had been inserted three months previously by a technique described by Coupland (1955). One

animal was killed after 7 days and the other after 14 days of drug treatment; the autografts were examined histologically and compared with similar autografts taken from saline-treated rats.

In the final experiment two groups of rats were taken—one of 9 and the other of 6 animals. Each member of the larger group received daily injections of TM 10 (10 mg./kg., s.c.) for 14 days, while the other 6 animals (controls) received injections of isotonic saline. At the end of this time 7 of the TM 10-treated, and all of the saline-treated, animals were killed. The adrenal glands of one of the TM 10-treated animals were set aside for histology, and those of the others dissected free of fat, weighed, and extracted for pressor amines. The extracts were subsequently assayed, as were similar extracts made from the glands of the control rats. The two surviving members of the TM 10-treated group were killed 6 and 14 days after stopping the injections and their adrenals examined histologically.

Histological Methods

The adrenals were bisected with a razor-blade—one-half being fixed in formol-dichromate (namely, neutral formaldehyde 5%, potassium dichromate 3%) for 18 hr., mordanted in 2% potassium dichromate for 24 hr., washed in water for 24 hr., dehydrated and embedded in paraffin. Sections were cut at thicknesses of 6 and 8 μ. The other half of the gland was placed in saturated potassium iodate for 8 hr., and then transferred to 5% formaldehyde for 24 to 48 hr., after which frozen sections were cut at a thickness of 30 μ. The eye-grafts were fixed in formol-dichromate for 12 hr., mordanted in dichromate for 24 hr., washed, dehydrated, paraffin-embedded, and sectioned at 6 μ. All the paraffin-embedded sections were stained with haematoxylin or haematoxylin and eosin. All the tissues were processed in an identical fashion in order to avoid any variations in staining, etc.

Formol-dichromate fixation results in a staining of cells containing either adrenaline or noradrenaline; potassium iodate treatment results in a selective staining of those elements which contain noradrenaline (Hillarp and Hökfelt, 1954).

Assay of Pressor Amines

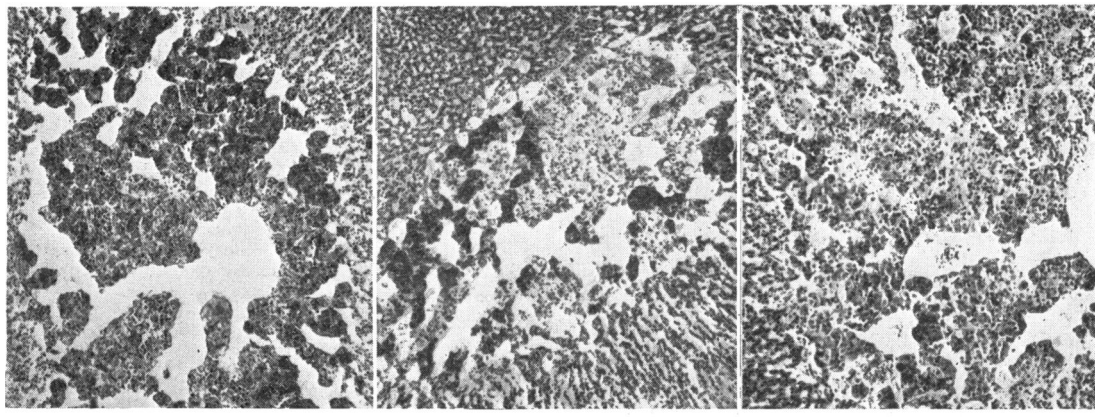
Both biological and colorimetric methods were used. The glands obtained from the TM 10-treated group of rats were pooled, weighed, and homogenized with 0.025 M-HCl, the *p*H adjusted to 5.5 by the addition of sodium acetate, placed in a boiling water bath for 3 min. and then cooled and centrifuged (Holland and Schümann, 1956). The adrenal glands of the saline-injected group were treated in the same way. The pressor amine content of the extracts obtained from the two groups of rats was estimated biologically by observing the effects on the blood pressure and acutely denervated nictitating membrane of a cat anaesthetized with chloralose and pre-treated (Coupland, 1953) with atropine sulphate (1 mg./kg., i.p.), cocaine HCl (5 mg./kg., i.m.), and mepyramine maleate (5 mg./kg., i.m.). Colorimetric estimations of the pressor amine content of the extracts were made by the method of Euler and Hamberg (1949).

RESULTS

Histological Observations

Effects of Insulin and TM 10 on the Adrenal Medulla.—The adrenals of rats killed five days after receiving convulsive doses of insulin were compared with those of rats similarly treated but given daily injections of TM 10, starting immediately after the insulin effects had subsided. Glands which were removed from animals five days after treatment with insulin alone showed a moderate chromaffin reaction (Fig. 1*a*) which was much greater than that observed in glands removed immediately after insulin treatment (Fig. 1*b*), thus indicating that some of the catechol amines lost were re-formed during the five-day period. The adrenal glands of rats treated with insulin followed by daily TM 10 for five days, on the other hand, showed only a faint chromaffin reaction (Fig. 1*c*). Thus the recovery of catechol amines appeared, from histological evidence, to be impeded by the daily administration of TM 10.

Comparison of the Effects of TM 10 and Choline Phenyl Ether Bromide on the Adrenal Medulla.—It was found, during the insulin experiments reported above, that TM 10 by itself was producing an effect on the chromaffin tissue. The effects of more prolonged administration of TM 10 upon the adrenals were therefore studied. Three groups of healthy rats were given daily injections of TM 10, isotonic saline, and choline phenyl ether bromide, respectively. The effects of choline phenyl ether bromide were studied, since it occurred to us that the action of TM 10 might be due to the weak nicotine-like stimulant properties of the drug. TM 10 has approximately 1/100th the stimulant activity of choline phenyl ether bromide (Willey, 1957) and so a daily dose of 0.1 mg./kg. of the latter drug was administered to the rats. Animals killed after seven days' treatment with TM 10 showed a moderately intense chromaffin reaction in the adrenals, and those killed after 14 days' treatment showed only a faint reaction (Fig. 2*a*). The chromaffin reactions of the choline phenyl ether- and saline-treated animals were, however, intense both at 7 and at 14 days (Fig. 2*b* and *c*). In all three groups of animals it was found that the staining properties of the noradrenaline-containing cells (as revealed by potassium iodate) were exactly similar to those of the whole medulla, described above—suggest-



a b c

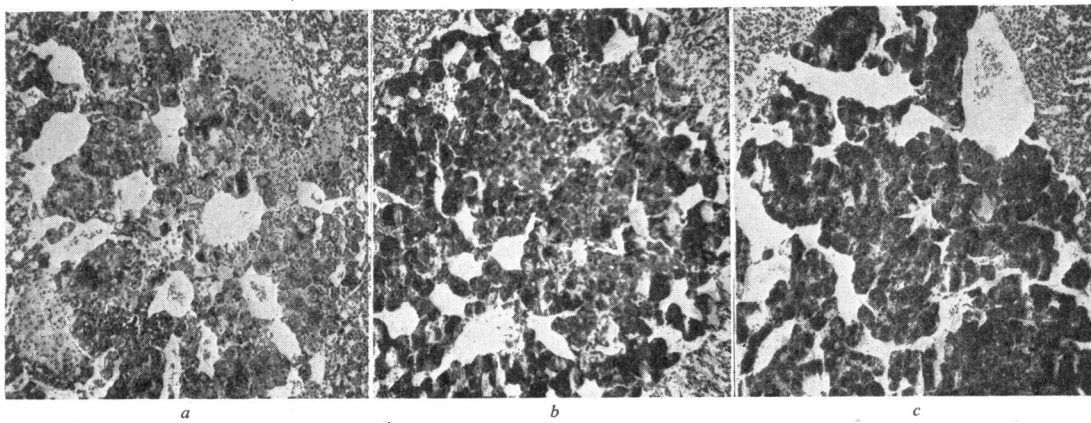
FIG. 1.—Medullary parts of rats' adrenals: (*a*) 5 days after receiving a convulsive dose of insulin; (*b*) immediately after three insulin convulsions; (*c*) 5 days after insulin convulsions and daily injections of TM 10. The restoration of chromaffin reaction after insulin shock is delayed by daily injections of TM 10. Formol-dichromate; haematoxylin; × 70.

ing that adrenaline and noradrenaline levels in the adrenal are equally influenced by TM 10.

These experiments suggest that TM 10 reduces the chromaffin reaction progressively, and by some other mechanism than nicotine-like stimulation of the medullary cells.

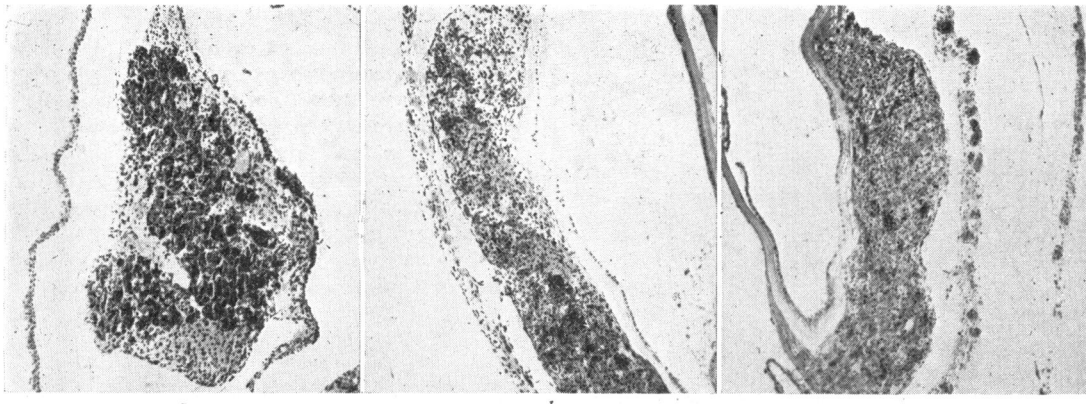
Effect of TM 10 on Autografts of Adrenal Medullary Tissue.—By comparison with control grafts (Fig. 3*a*), autografts obtained from rats treated with TM 10 showed a striking reduction in chromaffin reaction (Fig. 3*b* and *c*). The intensity of staining is less in the graft removed after 14 days' drug treatment than in the one removed after only seven days. Hence non-innervated, grafted, medullary cells appear to be affected by TM 10 in a similar way to those within the intact adrenal gland.

Changes in the Adrenal Medulla after Cessation of TM 10 Treatment.—Three rats, belonging to the group treated with TM 10 for 14 days (for the purposes of the quantitative experiment described below), were set aside for histological examination. One of these was killed one day after stopping the TM 10 injections; its adrenals showed, as expected, a very faint chromaffin reaction (Fig. 4*a*). Another animal was killed six days after stopping the treatment; its adrenals showed a moderate chromaffin reaction (Fig. 4*b*). The remaining rat was killed after 14 days and the chromaffin reaction was now indistinguishable from normal (Fig. 4*c*).



a b c

FIG. 2.—Rats' adrenal medullae after 14 daily injections of (*a*) TM 10, (*b*) choline phenyl ether bromide, (*c*) isotonic saline. Choline phenyl ether has little effect on the intensity of the chromaffin reaction, whereas TM 10 causes a marked reduction. Formol-dichromate; haematoxylin; × 70.



a b c

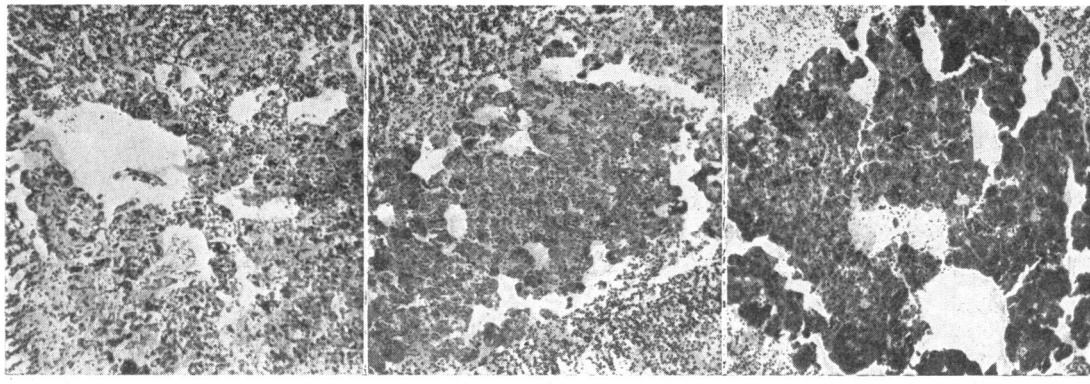
FIG. 3.—Autografts of rat adrenal medullae removed from the anterior chamber of the eye after daily injections of (*a*) isotonic saline for 14 days, (*b*) TM 10 for 7 days, (*c*) TM 10 for 14 days. TM 10 treatment reduces the intensity of the chromaffin reaction. The dark islands present in both graft and iris in (*c*) are intravascular collections of blood cells. Formol-dichromate; haematoxylin; ×70.

It is therefore concluded that, after TM 10 treatment under the conditions described, an interval of between 6 and 14 days is required for the restoration of the normal chromaffin-staining properties of the adrenal medulla.

Quantitative Estimation of the Degree of Depletion of Catechol Amines

Two groups, each of six rats, were used for the quantitative experiments. Animals in one of the groups were injected daily with TM 10 for 14 days (10 mg./kg., s.c.), whilst animals in the control group received daily injections of an equivalent volume of isotonic saline. At the completion of the treatment period the animals were killed, their adrenals removed, and the pooled weight of the glands determined for each group. For the TM 10-treated group, the total weight of the glands was 297 mg., whilst for the saline-treated group the weight was 285 mg.—weights close enough in agreement to exclude the occurrence of any significant atrophy or hypertrophy of the glands in rats receiving TM 10.

Extracts of the glands were made and the amounts of adrenaline and noradrenaline in each extract determined, biologically on the cat blood pressure and nictitating membrane, and colorimetrically, using solutions of pure (−)-adrenaline and (−)-noradrenaline, or mixtures of these, as standards of reference. Fig. 5 shows part of a record of the contractions of the nictitating membrane and blood pressure from the cat used for the estimation. The extract obtained from the TM 10-treated rats' adrenals contained only about



a b c

FIG. 4.—Rat adrenals removed at different times after cessation of a 14-day course of TM 10: (*a*) removed after 1 day; (*b*) after 6 days; (*c*) after 14 days. The chromaffin reaction returns slowly and is moderate after 6 days but intense at 14 days. Formol-dichromate; haematoxylin; × 70.

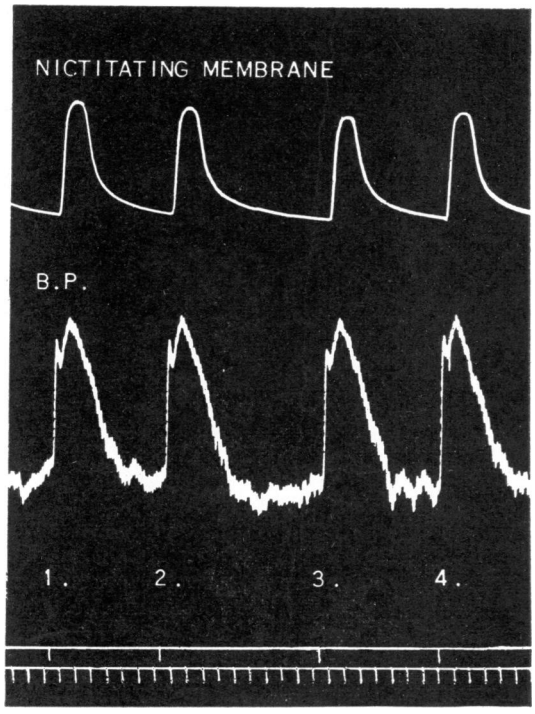


FIG. 5.—Cat anaesthetized with chloralose and pre-treated with atropine, cocaine, and mepyramine. Responses of nictitating membrane (upper record), and of blood pressure (lower record), to i.v. injections of (1) a mixture of 2·5 μg. adrenaline and 0·5 μg. noradrenaline; (2) 0·05 ml. of an extract of adrenals taken from saline-treated control rats; (3) 0·10 ml. of an extract of adrenals taken from rats treated for 14 days with TM 10 (10 mg./kg. daily); (4) a mixture of 2 μg. adrenaline and 1 μg. noradrenaline. Shows that the catechol amine content of the TM 10-treated rat adrenal extract is considerably less than that of the control. Time, 30 sec.

one-half the amount of pressor amines present in the control extract. The amine-content of the two extracts was then estimated colorimetrically, and, from the results shown in Table I, it can be seen that there is satisfactory agreement between the results afforded by the two methods. The amine content is expressed as μg./g. adrenal gland tissue, and average values for the biological and colorimetric results are shown.

It is interesting to note, from the results given in Table I, that the reduction in adrenaline- and noradrenaline-content of the adrenals, resulting from treatment of the rats with TM 10, affects both amines in the same proportion so that the ratio of adrenaline to noradrenaline in the glands remains practically unchanged at about 3 to 1. Thus it would seem improbable that TM 10 affects, in any way, the processes of methylation within the adrenals.

General Effects of TM 10 on Rats

None of the animals treated with TM 10 died, nor did they exhibit loss of weight or signs of ill-health. There were no pathological signs at autopsy. There was, however, a consistent circumscribed loss of hair immediately over the site of each injection which followed about two days after the injection. The reason for this was not obvious, for the drug was administered aseptically on all occasions; no hair-loss occurred in the saline- or choline phenyl ether-treated rats.

DISCUSSION

Quantitative assay of the pressor amine content of the adrenal glands of rats treated with TM 10 daily for 14 days has shown that the amount of adrenaline and noradrenaline present is only about one-half that found in saline-treated control animals. Histological observations on sections cut from the suprarenals support these results by demonstrating a marked reduction in the intensity of the chromaffin reaction of the medullary cells. Reduction in catechol amines is histologically recognizable as early as the fifth day of treatment, and becomes progressively more marked as treatment continues.

TM 10 may act in one of three different ways, viz.: (1), the drug may interfere with biosynthesis by blocking one or more chemical stages in the elaboration of catechol amines; (2), it may have a stimulating action on some part of the nervous system, resulting in increased discharge of splanchnic nerve impulses; (3), it may act as a direct stimulant of the medullary cells whereby adrenaline, noradrenaline, or their precursors, are liberated into the general circulation and the gland thus exhausted of these substances.

Of the three possibilities, it would seem that the first is better supported by the experimental facts than are either the second or third; for, though TM 10 has weak nicotine-like stimulant actions (Hey and Willey, 1954; Willey, 1957) which might result

TABLE I

EFFECT OF TM 10 ON CATECHOL AMINE-CONTENT OF RAT ADRENALS

Amine	Quantity Estimated (μg./g. Gland Tissue)				% Adrenaline in Total
	Bio-assay	Colorimetric	Average	Total Catechols	
Control Group:					
Adrenaline	808	735	771	1,029	75
Noradrenaline ..	267	250	258		
TM 10-treated Group:					
Adrenaline	402	382	392	536	73
Noradrenaline ..	134	155	144		

in direct stimulation of medullary cells, it is unlikely that these play a significant part in the depletion process, since treatment of rats for two weeks with daily injections of choline phenyl ether bromide—a compound with powerful nicotine-like stimulant actions—does not result in histological evidence of catechol amine depletion. Furthermore, the possibility that TM 10 might exert its effect centrally, causing increased splanchnic nerve activity, is ruled out by the experiments on rats with chromaffin tissue grafted into the anterior chamber of the eye. Such grafts are entirely without nervous connexions (Coupland, unpublished observations); nevertheless, they showed marked histological signs of amine depletion in the TM 10-treated rats, whereas grafts obtained from saline- or insulin-treated animals showed no detectable changes (Coupland, unpublished observations).

It is of special interest to recall the observations of Udenfriend and his co-workers, who were able to estimate (by pre-treating rats with ^{14}C-labelled tyrosine or phenylalanine) the approximate time taken for half a given amount of radioactive adrenaline or noradrenaline to disappear from the store of these catechols in the suprarenals. Their results indicated a half turnover time for adrenaline in the rat suprarenal of about 9 days (Udenfriend, Cooper, Clark, and Baer, 1953) and, in later experiments, a half turnover time for both adrenaline and noradrenaline of about seven days (Udenfriend and Wyngaarden, 1956).

On the basis of Udenfriend's work it could, therefore, be predicted that if the biosynthesis of catechol amines in the rat was blocked completely, it would take from 7 to 9 days for the total suprarenal amines to fall to one-half their normal level. Under the conditions of our experiments, where the animals received only one dose of the drug per 24 hr., it is unlikely that biosynthesis could have been blocked sufficiently to achieve the maximum rate of depletion thus predicted. Nevertheless, though the similarity may be coincidental, the remarkably close agreement between the time relationships as revealed by the experiments of Udenfriend *et al.* (1953), and those of the present work—in which a half-depletion of catechol amines occurred after 14 days' treatment with TM 10—suggests to us that the mode of action of TM 10 is one of interference with biosynthesis, rather than one of liberation of stored amines from the cells.

The effects of TM 10 on the rat suprarenal appear to be fully reversible; histological signs of restoration of catechol amines made their appearance by the sixth day after the cessation of TM 10-treatment, and restoration appeared complete by the 14th day. These observations accord fully with the information yielded by Hökfelt's (1951) studies on the re-formation of adrenal catechol amines following depletion by insulin: he showed that about six days were required for full restoration, thus indicating that the rate of synthesis of catechol amines in the rat adrenal medulla is slow.

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