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THE PROLONGATION OF RESERPINE-INDUCED CARDIAC NOREPINEPHRINE DEPLETION BY METABOLIC INHIBITORS*

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Abstract—Levels of cardiac norepinephrine (NE) in the rat were determined at various times after the intraperitoneal administration of 2 mg/kg reserpine phosphate. The rate of repletion of NE was decreased by the administration of certain metabolic inhibitors [thioacetamide; 2,2-dichloro-*N*-(β-hydroxy-α-(hydroxymethyl) -*p*-methylsulfonyl-phenethyl) acetamide; β-diethylaminoethyl-diphenylpropyl acetate (SKF 525-A); actinomycin D] in proper dosage schedules. None of these agents significantly affected the endogenous NE concentration in the heart of the nonreserpinized rat. In animals pretreated with phenobarbital prior to reserpine, the levels of cardiac NE returned to normal more rapidly than did those of animals receiving no prior barbiturate. SKF 525-A decreased the levels of ^{3}H-reserpine found in the heart and liver, and did not affect the concentration of ^{3}H-reserpine in the carcass of animals 4 days after 2 mg/kg ^{3}H-reserpine.

Thioacetamide did not alter the capacity of the heart to retain tritiated NE after i.v. administration. Actinomycin D did not interfere with the rate of NE synthesis in the heart, nor did it change the characteristics of the NE uptake process of isolated perfused hearts from reserpinized animals. Repletion of adrenal NE in reserpinized animals was not affected by administration of actinomycin D. It was concluded that the slow repletion of the cardiac NE after reserpine administration may be due to the necessity for the synthesis of new NE storage sites in the heart, and that the metabolic inhibitors retard the return of normal NE levels by inhibiting the synthesis of these storage sites.

SOON AFTER Holtzbauer and Vogt[1] demonstrated that a decrease in tissue catecholamines occurs after reserpine administration, many workers[2–5] showed that the levels of residual amines in many organs remained low for several days. No adequate explanation has been offered for the long time required for restoration of tissue catecholamine levels to normal after reserpine.

Initial studies by Hess *et al.*[6] using a fluorimetric assay, and by Sheppard *et al.*[7] using ^{14}C-labeled reserpine, indicated that reserpine was undetectable in tissues within a few hours after its administration. Subsequent studies[8, 9] with randomly labeled ^{3}H-reserpine showed that reserpine is detectable throughout and after the time when objective behavioral changes are present. However, no correlation has been shown to exist between levels of reserpine or known metabolites in the brain and manifestations of its action on the central nervous system. Hence, it is possible that no correlation

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exists peripherally between levels of reserpine and blockade of catecholamine storage.

Most studues[10, 11] have indicated that one of the acute effects of reserpine, i.e. depletion of catecholamines, is due to an inhibition of an uptake mechanism which functions to maintain a high concentration of catecholamines in the terminals of adrenergic neurons. Jonasson *et al.*[12] have elaborated on the concept, suggesting that the affected transport process is in the neuronal vesicle, and that the inhibition is of a competitive type. Alternative explanations of the depletion produced by reserpine have been offered. An increase in the levels of monoamine oxidase or catechol-*O*-methyl transferase resulting in enhanced catabolism of catecholamines as a possible mechanism has been eliminated by the work of Kopin *et al.*[13] Other studies[14–16] indicate that an inhibition of catecholamine synthesis by reserpine is unlikely as an explanation of amine depletion by this drug.

It is possible that the long-lasting effects of reserpine are the consequence of a persistent change in neuronal vesicles due to either prolonged binding of reserpine or a metabolite to the granules, or to irreversible inactivation of the granular norepinephrine (NE) uptake process. Hence reestablishment of normal levels of amines occurs only when the affected granules are replaced by new ones. If this were correct, it should be possible to study the rate of synthesis of new neuronal vesicles and determine if this rate were comparable to the rate of return of catecholamine levels to normal. Unfortunately, determinations of the rate of synthesis of protein or ribonucleic acid (RNA) of the microsomal fraction of rat heart indicated that these rates were very slow, a finding not unlike those of Khan and Wilson,[17] who reported a long (15 days) half-life of crude "microsomal" fractions of rat brain and other tissues. However, indirect evidence for storage site resynthesis after reserpinization might be obtained if one could inhibit such resynthesis and prolong the reserpine-induced depletion of catecholamines. Therefore, different inhibitors of protein or RNA synthesis were examined for their effects on the reserpine-induced depletion of cardiac catecholamines in the rat.

METHODS

Handling of animals and preparation of tissues. Male rats of the Holtzman strain weighing 60–100 g were employed in these studies. Since thioacetamide, the methylsulfonyl derivative of chloramphenicol, actinomycin D, and SKF 525-A significantly reduced food consumption and weight gain, a paired feeding technique was used in experiments utilizing these agents. The amount of food eaten by each experimental animal on one day was given to his initially weight-matched control the following day. In order to measure food consumption accurately, both experimental and control animals received pulverized Purina rat biscuits in a container which permitted only the insertion of the animal's head. Animals were individually housed under environmental conditions as nearly identical as possible.

Animals were killed by a blow on the head and immediately decapitated. The hearts were removed, blotted free of blood on gauze pads, weighed, and analyzed for NE according to the procedure of Anton and Sayre.[18] Cardiac *dl*-7-^{3}H-norepinephrine (^{3}H-NE) content was determined by the method of Whitby *et al.*[19] Subcellular fractionation of homogenates of rat ventricle prepared in 0·075 M potassium phosphate buffer (pH 7·5) was accomplished by an initial centrifugation at 5000 *g* for 5 min yielding a sediment designated IS. The supernatant fluid was centrifuged at 105,000 *g*

for 60 min. This yielded a second sediment (HSSF) and a final supernatant fraction (S).

In animals which were examined for adrenal catecholamines, the adrenal glands were exposed by an abdominal incision, the capsules incised, and the medullae expressed by using a pair of fine forceps. The tissue so obtained was placed in 0·075 M potassium phosphate buffer (pH 7·5) and homogenized at 4°. The protein concentration in an aliquot of this homogenate was estimated by the method of Lowry *et al.*[20] The resultant value was corrected for the slight interference caused by the large amounts of catecholamines present. Cardiac concentrations of NE or ^{3}H-NE were expressed on a wet weight basis. The concentration of ^{3}H-NE in each subcellular fraction was expressed as a per cent of the total in all fractions.

Uptake of NE by the heart. The uptake of NE by isolated rat hearts was studied in essentially the manner described by Iversen,[21] employing Nasmyth's solution,[22] which was modified to contain calcium in a concentration of 1·275 mM. When employed, ^{3}H-NE was present in the perfusion fluid at a concentration of 9 mμc/ml, double the amount utilized by Iversen. The sp. act. of the ^{3}H-NE was reduced by addition of *dl*-norepinephrine hydrochloride to achieve final concentrations of NE(free base) ranging from 20–300 ng/ml. ^{3}H-NE was extracted and estimated by the method of Whitby *et al.*[19] Deaminated metabolites were separated from the final aluminum oxide eluate by the procedure of Kopin *et al.*[23] Rats were injected with 1000 units of heparin (i.p.) 5 min before being killed. After removal from the animal, the hearts were perfused by the Langendorff technique at a perfusion pressure of 90 cm water. After perfusion with Nasmyth's solution, modified as described above, hearts were perfused for 5 min with a solution containing ^{3}H-NE for various periods of time, and finally for 3 min with modified Nasmyth's solution to wash the ^{3}H-NE out of the extracellular space. By means of a three-way stopcock, the perfusing solution was rapidly changed from one perfusing fluid to another. Since the initial velocity of ^{3}H-NE uptake for high NE concentrations in the perfusion fluid must be obtained by extrapolation to zero time, several determinations were made after perfusion for several different time intervals to permit visual extrapolation to initial uptake velocities. A plot of the reciprocal of the initial velocity of ^{3}H-NE uptake against the reciprocal of the concentration of NE in the perfusing solution was made and the line of best fit was obtained by the method of weighted least squares proposed by Wilkinson.[24] A Fortran program written by Cleland[25] was employed with a Control Data 1604 computer to estimate kinetic parameters and their S.E.'s.

Estimation of the rate of synthesis of NE. Studies of the rate of exponential decline in ^{3}H-NE concentration after its administration to rats were similar to those described by Montanari *et al.*[26] However, the ^{3}H-NE employed in these studies had a higher sp. act. (8·35 c/m-mole) than that used by these authors. Each rat received 40 μc/kg of ^{3}H-NE in 0·4 ml of 0·9% saline via the tail vein. Groups of four animals receiving only ^{3}H-NE were killed at 18, 27, 36, and 48 hr and the ventricular ^{3}H-NE was estimated by the method of Whitby *et al.*[19] Similar studies were performed with animals which had received 100 μg/kg of actinomycin D 12 hr prior to administration of ^{3}H-NE. Since the amount of administered NE was insufficient to alter the concentration of NE in the heart and since this dose of actinomycin D did not significantly affect the level of endogenous NE, specific activities were not determined. Rather the data were calculated as dpm/g wet wt. of ventricle, the same method of calculation employed by Montanari *et al.*[26]

Measurement of the rate of disappearance of tritiated reserpine. Reserpine, labeled with tritium on the ring of the trimethoxybenzoic acid portion (TMBA) of the molecule (^{3}H-reserpine), was purchased from New England Nuclear Corp. Its purity was established chromatographically by means of the system described subsequently. This material was supplied as a solution in ethanol or benzene and had a sp. act. of 277 mc/m-mole. When supplied in ethanol, the ethanol was not removed, but when supplied in benzene vehicle, the benzene was removed by evaporation under a stream of nitrogen in the dark. The sp. act. was decreased to 152·4 mc/m-mole by the addition of unlabeled reserpine. Sufficient vehicle (Serpasil-injectable solution, placebo, Ciba) was added to provide a solution which contained 50 μc or 0·2 mg/ml. All animals in these studies received 10 ml/kg of the latter solution i.p.

The separation of ^{3}H-reserpine from tissue samples was accomplished by a modification of the procedure of Sheppard *et al.*[27] Tissues were homogenized in 10 ml purified acetone, transferred with two 2-ml washings to a centrifuge tube, and centrifuged to remove tissue fragments. The supernatant fluid was combined with 8 ml of 8% acetic acid and the mixture was extracted with 10 ml of normal hexane. This and subsequent extractions were performed in stoppered glass centrifuge tubes shaken at 200 oscillations per min for 5 min. The phases were separated by centrifugation at 1500 rpm for 5 min in an International centrifuge, model EXD, with a No. 250 head. The hexane phase was removed and saved, and the aqueous phase was extracted with another 10 ml hexane. The two hexane extracts were combined and extracted with 5·0 ml of 4% acetic acid. The aqueous extracts were combined and extracted successively with three portions of 7 ml chloroform each. The chloroform extracts were combined and evaporated to dryness under reduced pressure. The dry residue was removed from the flask with five 1-ml portions of chloroform which were combined in a 20-ml vial. By using this extraction procedure, 99·3 per cent of chromatographically pure ^{3}H-reserpine (1 μc/mg in 10 ml acetone) is found in the chloroform fraction.

After evaporating the concentrated chloroform extract under a stream of nitrogen in the dark, 0·5 ml absolute ethanol, 5 μg reserpine phosphate, and 50 μg TMBA were added. Twenty-five–100 μl of the ethanol mixture was chromatographed by thin-layer chromatography with MN cellulose powder 300 G/ECTEOLA of 10μ particle size containing 10% $CaSO_4.1/2\ H_2O$, as supplied by Macherey, Nagel, and Co. (Duren, Germany). Five μg reserpine and 50 μg TMBA were chromatographed as reference markers on every plate. The relative R_f of TMBA was 0·5–0·6 and that of reserpine was 0·9 in a solvent mixture containing methanol:butanol:pyridine:water in the proportion 1:1:1:1 (v/v). By using u.v. light, the areas occupied by the reference compounds were used to select the areas of the developed chromatogram which contained the labeled reserpine and TMBA in each sample. A segment between these two areas was also removed to assure successful separation of the reserpine and TMBA. After removal of the areas from the chromatograms, the radioactive compounds were eluted with absolute ethanol. Initial elution with 3·0 ml ethanol was successful in removing 93 per cent and a second elution removed 4·9 per cent of the radioactivity in each area. Therefore, in all experiments three elutions were used. The R_f values of nonhydrolyzed metabolic products of reserpine were not determined in this system; therefore, it is possible that activity attributed to reserpine may also represent such products.

To estimate the amount of tritiated reserpine and TMBA present in each sample,

the total tritium content in each chloroform fraction was multiplied by the per cent tritium present as reserpine or TMBA, as determined by TLC.

When the total rat carcass was analyzed for residual reserpine, the above procedure was altered somewhat. The animal was killed, the tail removed, and the balance of the carcass minced in a Waring–Blender, model CB-5, with 300 ml of reagent grade acetone at low speed for 1½ min, and then at medium speed for 30 sec. The resulting slurry was filtered through gauze pads, washed with acetone, and the filtrate diluted to 500 ml. The filtrate was centrifuged at 500 *g* for 12 min in a refrigerated Lourdes centrifuge using the VRA head. The supernatant fluid was evaporated under reduced pressure at 50° to a volume of 50 ml, divided into five 10-ml portions, and processed as previously described.

Estimation of radioactivity. All measurements of radioactivity were made by using a dioxane phosphor solution[28] and a Packard liquid scintillation spectrometer. Values were corrected for quenching by the use of internal standards.

Materials. Reserpine phosphate was supplied by Dr. A. J. Plummer, Ciba Pharmaceutical Co.; the methylsulfonyl analog of chloramphenicol (2,2-dichloro-*N*-(β-hydroxy-α-(hydroxymethyl)-*p*-methylslufonylphenethyl)acetamide) by Dr. A. S. Weisberger, Western Reserve University Medical School; SKF 525-A (2-diethylaminoethyl-2,2-diphenylvalerate HCl) by Dr. G. E. Ullyot, Smith Kline & French Laboratories; actinomycin D by Merck Sharp & Dohme. Thioacetamide was purchased from Fisher Scientific Co.; 3,4,5-trimethoxybenzoic acid from K & K Laboratories, Inc.; *dl*-7-^{3}H-norepinephrine and ^{3}H-reserpine from New England Nuclear Corp.

Statistics. Statistical significance of observed differences was determined by means of either a paired or unpaired *t*-test, as dictated by the comparison made.[29] When paired observations are analyzed for significance in the text, the symbol $s\text{-}_d$ represents the S.D. of the difference, and the symbol *df* represents the appropriate degrees of freedom.

RESULTS

Effects of various metabolic inhibitors on endogenous cardiac NE

None of the agents (thioacetamide, the methylsulfonyl analog of chloramphenicol, actinomycin D, SKF 525-A, phenobarbital sodium) used in these studies significantly altered endogenous NE levels in rat ventricles in the dosage regimens employed (Table 1). A possible effect on distribution of NE among subcellular storage sites which is not reflected in an alteration of total NE concentrations must be considered. In the case of thioacetamide, however, this did not appear to be true, at least in those fractions which were examined.

Effects of various agents on repletion of cardiac NE after depletion by reserpine

The slow rate of repletion of NE in the heart of the rat after i.p. administration of reserpine is illustrated in Fig. 1. Reserpinized (2 mg/kg, i.p.) animals that were totally fasted exhibited lower concentrations of cardiac NE 4 days after reserpine than did non-fasted animals (non-fasted − fasted = 0·20 μg NE/g; $s\text{-}_d = 0{\cdot}06$ for 9 *df*; $P < 0{\cdot}05$). Since all drugs studied for their inhibitory effects on the repletion of reserpine-induced depletion of NE significantly decreased food intake, the paired feeding technique was utilized except in the case of animals pretreated with phenobarbital.

Daily administration of thioacetamide, 50 mg/kg i.p., for 10 days before and subsequent to reserpine altered the course of cardiac NE repletion (Fig. 2). On the day of reserpine administration, the animals received both drugs by the same route but separated by several hours. At 2 and 4 days, but not at 8 days, after reserpine there was

TABLE 1. THE EFFECTS OF VARIOUS AGENTS ON THE CONCENTRATION OF NOREPINEPHRINE IN THE VENTRICLES OF THE RAT HEART

Agent*	No. of animals	Dose (mg/kg/day)	No. of daily doses	Norepinephrine (μg/g $\pm$ S. E.)
None	6			1·01 $\pm$ 0·05
Methylsulfonyl chloramphenicol	4	200	4	1·11 $\pm$ 0·05†
SKF 525-A	6	50	4	1·03 $\pm$ 0·12†
SKF 525-A	9	50¶		0·98 $\pm$ 0·06‡
Actinomycin D	6	0·1	1	0·91 $\pm$ 0·04§
Actinomycin D	6	0·1	1	0·88 $\pm$ 0·06‖
Phenobarbital	8	40	4	0·96 $\pm$ 0·08†
Thioacetamide	12	50	10	1·15 $\pm$ 0·06†

* Administered i.p.
† Determined 24 hr after last injection of agent.
‡ Determined 8 hr after last injection of agent.
§ Determined 48 hr after injection of agent.
‖ Determined 96 hr after injection of agent.
¶ This dose was administered at 0, 12, 24, 36, 44, 52, 60, 68, and 76 hr.

a significant impairment of repletion. The mean difference in NE concentration at 4 days after reserpine administration between treated (reserpine + thioacetamide) and control animals (reserpine) was greater when animals were allowed free access to food (0·24 μg NE/g; $s\text{-}_{\bar{d}} = 0{\cdot}07$ for 10 *df*; $P < 0{\cdot}01$) than when they were pair fed (0·16 μg NE/g; $s\text{-}_{\bar{d}} = 0{\cdot}04$ for 8 *df*; $P < 0{\cdot}05$).

Intraperitoneal administration of the methylsulfonyl derivative of chloramphenicol (0·2 g/kg) daily for 7 days before reserpinization and thereafter until the animals were killed, significantly reduced the degree of NE repletion normally occurring 4 days after reserpine administration (mean difference = 0·15 μg NE/g; $s\text{-}_{\bar{d}} = 0{\cdot}06$ for 6 *df*;

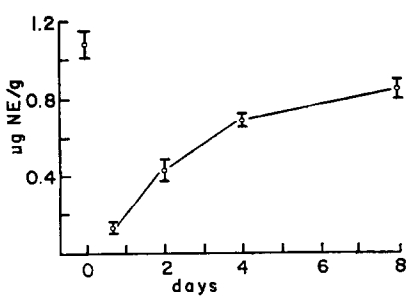

FIG. 1. Repletion of norepinephrine in the ventricles of the rat heart after administration of reserpine. Rats were killed and cardiac NE determined at the indicated times after injection of 2·0 mg/kg reserpine phosphate, i.p. Each point on the curve represents the mean of at least 6 animals. Vertical lines at each point indicate the magnitude of the S.E.M. The value at zero time is for untreated animals.

$P < 0.05$). On the day of reserpinization the injections were again separated by several hours.

Both thioacetamide and the methylsulfonyl derivative of chloramphenicol required a prolonged period of administration prior to reserpinization. It was of some interest therefore to see if a more acutely acting substance, an inhibitor of ribonucleic acid

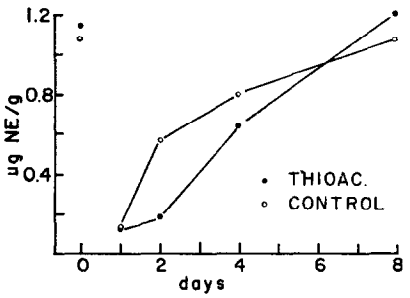

FIG. 2. Effect of thioacetamide on repletion of cardiac NE in the rat ventricles after its depletion by reserpine. Animals were killed and NE concentrations determined at the indicated times after administration of reserpine phosphate (2·0 mg/kg, i.p.) to both thioacetamide-pretreated and control animals. At zero time, the two points represent the means of at least 4 animals which had received thioacetamide (50 mg/kg/day, i.p.) as described in the text and their pair fed controls. A statistically significant difference ($P < 0.05$) between the two groups was observed at 2 days ($s\text{-}d$, 0·037) and 4 days ($s\text{-}d = 0.019$) after reserpine, using the t-test appropriate to paired observations.

synthesis, actinomycin D, could also affect the cardiac repletion of NE after its depletion by reserpine. Fig. 3 shows the alteration of repletion caused by the i.p. administration of 100 µg/kg of actinomycin D 2 days after reserpine. This produced a significant inhibition of repletion at 4 and 6 days after reserpine. Administration of a second dose of actinomycin D at 4 days after reserpine did not further increase the magnitude of the difference at 6 days after reserpine from that seen with the single dose of actinomycin D 2 days after reserpine.

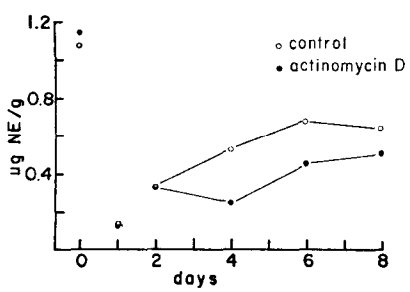

FIG. 3. Alterations of cardiac NE repletion by actinomycin D after reserpine administration. Animals were killed and NE values determined at the indicated times after administration of reserpine phosphate (2·0 mg/kg, i.p.). Control animals received reserpine only; actinomycin D (100 µg/kg, i.p.) was administered to the experimental group 2 days after reserpine. The two values at 4, 6, and 8 days after reserpine represent the means of 5 animals which had received actinomycin D and their pair fed controls. Controls were pair fed from the time the experimental animals received actinomycin D. The value on day 1 is taken from Fig. 1. The values are significantly different at 4 days (control − treated = 0·28; $P < 0.01$; $s\text{-}d = 0.07$), and at 6 days (control − treated = 0·25; $P < 0.05$; $s\text{-}d = 0.11$), but not at 8 days.

The rate of disappearance of reserpine

Sladek and Mannering[30] showed that thioacetamide inhibits *N*-demethylation of ethylmorphine and 3-methyl-4-monomethylaminoazobenzene by liver microsomes of normal rats. Sheppard and Tsien[31] had previously shown that particulate fractions prepared from homogenates of guinea pig liver metabolize reserpine *in vitro*. Hence, the possibility that thioacetamide might exert its effects on repletion of NE by interfering with the metabolism of reserpine in the liver was considered. A similar explanation of the action of the methylsulfonyl analog of chloramphenicol also is possible. If the liver microsomal system is responsible for the metabolic inactivation of reserpine, one might expect that animals with increased activity of the system would catabolize reserpine more readily and replenish their NE stores more rapidly. To test this possibility, phenobarbital, an agent known to increase the activity of the hepatic microsomal enzyme system in rats,[32] was administered to animals prior to reserpinization. Fig. 4

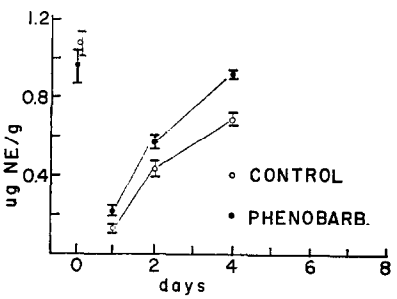

FIG. 4. Effect of pretreatment with phenobarbital on repletion of cardiac NE after reserpine. Animals were killed and NE determined at the times indicated after administration of reserpine phosphate (2·0 mg/kg, i.p.) at zero time. Animals pretreated with phenobarbital sodium received 40 mg/kg/day for 4 days prior to reserpine. Both groups of animals were fed *ad libitum*. Values represent the mean ± S.E. Statistically significant ($P < 0{\cdot}05$) differences between control and phenobarbital-treated animals were observed at 2 and 4 days after reserpine by using the *t*-test appropriate to unpaired observations. Each point represents the mean of at least 6 animals.

indicates that in animals which had received daily i.p. injections of sodium phenobarbital (40 mg/kg/day) cardiac NE repletion occurs earlier, but at a rate approximating that of control hearts. This may be the result of less complete depletion in the animals receiving phenobarbital because of more rapid metabolic inactivation of reserpine. When administration of phenobarbital was begun on the day reserpine was injected, no effect on NE repletion was observed 4 days later (control − treated = 0·02 μg NE/g).

Inhibition of the metabolism of reserpine might be expected to have an effect opposite to that seen with phenobarbital pretreatment. Even thought the exact reaction inactivating reserpine is not known, a known inhibitor of the hepatic microsomal drug-metabolizing system, SKF 525-A was employed to determine if it might also cause an inhibition of NE repletion after reserpine treatment. When this inhibitor was given i.p. in a dose of 50 mg/kg 45 min before administration of reserpine, it did not significantly alter the degree of repletion of NE 4 days after reserpine (control − treated = 0·10 μg NE/g; $s\text{-}_{\bar{d}} = 0{\cdot}08$ for 12 *df*; $P > 0{\cdot}05$). However, daily injections of SKF 525-A (50 mg/kg/day) after reserpine administration did significantly inhibit NE repletion at 4 days after reserpine (control − treated = 0·16 μg NE/g; $s\text{-}_{\bar{d}} = 0{\cdot}07$

for 13 *df*; $P < 0.05$). Further, a more intensive dosage schedule to decrease possible inductive effects due to SKF 525-A increased the magnitude of the difference in cardiac NE values observed at this time (control − treated = 0·27 μg NE/g; $s\text{-}_{\bar{d}} = 0.07$ for 8 *df*; $P < 0.01$). Animals in this latter group received SKF 525-A (50 mg/kg) 12, 24, 36, 48, 60, 72, 80, and 88 hr after reserpine. Although it might be concluded from these studies that the observed effects of SKF 525-A are the result of inhibition of the metabolism of reserpine, Neubert and Timmler[33] have shown an inhibition of ^{14}C-alanine incorporation into liver protein *in vitro* by SKF 525-A, an effect which they concluded was unrelated to inhibition of hepatic microsomal drug metabolism. Thus, it is possible that the inhibition of NE repletion associated with SKF 525-A administration is not due to interference with reserpine metabolism.

It thus became necessary to determine whether SKF 525-A is capable of inhibiting the metabolism of reserpine. This might be evaluated by determining the rate of labeled reserpine disappearance from the heart, the liver, or indeed from the body as a whole in control animals and in animals receiving the more intensive SKF 525-A dosage schedule described above. Accordingly, ^{3}H-reserpine was administered to two groups of animals, one receiving only reserpine and the other reserpine and the more intensive SKF 525-A dosage schedule. The amounts of ^{3}H-reserpine remaining in these two groups of animals 4 days after treatment with 2·0 mg/kg of ^{3}H-reserpine (25 μc/mg) are shown in Table 2. Since significantly less reserpine was found at 4 days

TABLE 2. EFFECT OF SKF 525-A AND ACTINOMYCIN D ON THE RATE OF DISAPPEARANCE OF TRITIATED RESERPINE FROM THE RAT

Tissue analyzed	Concentration of reserpine (ng/g wet wt.* 96 hr after reserpine		
	Control†	SKF 525-A‡	Actinomycin D§
Entire animal	1·1 ± 0·8 (5)‖	1·2 ± 0·2 (3)	
Heart	3·4 ± 0·1 (7)	1·6 ± 0·2 (6)	2·2 ± 0·2 (5)
Liver	3·3 ± 0·1 (7)	1·3 ± 0·4 (6)	

* Calculated by considering all tritium isolated as reserpine to be authentic reserpine.

† Reserpine, 2 mg/kg, i.p. at 0 hr.

‡ Administered in a dose of 50 mg/kg, i.p., at 12, 24, 36, 48, 56, 64, 72, 80, and 88 hr after reserpine (2 mg/kg, i.p.).

§ Administered in a dose of 100 μg/kg, i.p., 48 hr after reserpine (2 mg/kg, i.p.).

‖ Figures in parentheses indicate number of animals on which each mean is based.

in both the hearts and livers of animals treated with SKF 525-A than in controls, it is unlikely that the mechanism of action of this agent can be ascribed to inhibition of metabolism of reserpine and hence prolongation of its action. In the whole animal, the amount of residual ^{3}H-reserpine present in the SKF 525-A-treated animal was not significantly different from control values.

When the experiment was repeated with animals which received actinomycin D (100 μg/kg, i.p.) 2 days after the ^{3}H-reserpine, no increase in cardiac ^{3}H-reserpine concentration was observed 4 days after reserpinization (Table 2).

The effects of actinomycin D and thioacetamide on NE synthesis, retention and uptake

The possibility that SKF 525-A, thioacetamide, actinomycin D, and the methylsulfonyl analog of chloramphenicol alter the dynamics of synthesis or uptake of NE and that such alterations may account for the prolongation of reserpine-induced depletion of this amine, were considered and examined by using actinomycin D and thioacetamide.

Montanari *et al.*[26] have analyzed the compartment and pool hypotheses advanced by numerous workers and have concluded that in the heart, regardless of the true nature of storage forms, the rate of NE synthesis varies with the slope of the exponential phase of decline in sp. act. of NE after a single injection of ^{3}H-NE having a high sp. act. Although the method of estimating the rate of NE synthesis is indirect, it would reflect synthesis of NE from all precursors which can be taken up from the circulation. These authors emphasized that this method is valid only when the total amount of NE present is not changing. Thus we were limited to observing the decrease in radioactivity in control animals and in animals which had received actinomycin D 18 hr previously, but were not reserpinized. This decrease in radioactivity is then proportional to the rate of NE synthesis in these experimental groups. As shown in Fig. 5,

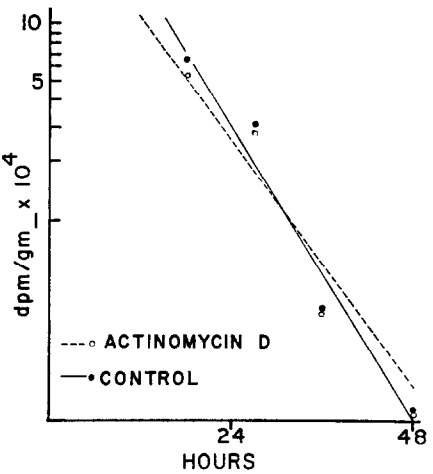

FIG. 5. Rate of disappearance of ^{3}H-NE from the rat ventricle. All animals received 4 μc of *dl*-^{3}H-NE, i.v., at zero time, and were killed at the times indicated. ^{3}H-NE remaining in the ventricle was analyzed as described in the text. Each point represents the mean of 4 values. Animals which received actinomycin D were injected with this agent 12 hr before receiving the ^{3}H-NE. Slopes were calculated by the method of least squares applied to the individual values in each group. There was no significant difference in the slopes calculated for the two groups.

there is no significant difference between the slopes of these two rates of label disappearance and hence, if the assumptions are valid, there is no alteration in the rate of NE synthesis in nonreserpine-treated animals which received actinomycin D. Admittedly, it is possible that some alteration in rate of NE synthesis occurs if the assumptions are not valid, or if actinomycin D has a different action in the reserpinized animal than it does in normal rats.

The possibility that thioacetamide could be altering the repletion of NE by interfering with the capacity of the cardiac nerve terminals to retain NE removed from the

circulation was examined. Aniamsl treated for 10 days with thioacetamide (50 mg/kg) had a concentration of cardiac ^{3}H-NE equal to control animals when examined 2 hr after i.v. administration of the labeled amine (50 μc/kg; 10·0 c/m-mole). No alteration was observed in the distribution of labeled amine among the subcellular compartments separated as described under Methods.

Actinomycin D was examined for its capacity to alter uptake mechanisms for NE in the rat ventricle. Iversen[21] recently studied the uptake of catecholamines by rat hearts and demonstrated that variations of rate of NE uptake seen with various concentrations of NE in the pefusing medium are described by Michaelis-Menten kinetics. If actinomycin D were altering the NE repletion rate by interfering with the uptake process, this might be reflected in an alteration of the kinetic characteristic for the process. It is evident from Fig. 6 there was no difference between these parameters in hearts obtained from rats 4 days after reserpinization and those of rats which also received actinomycin D 2 days after reserpine. Each point in this figure represents the mean of from 6–10 hearts. A total of 36 hearts was used in each group.

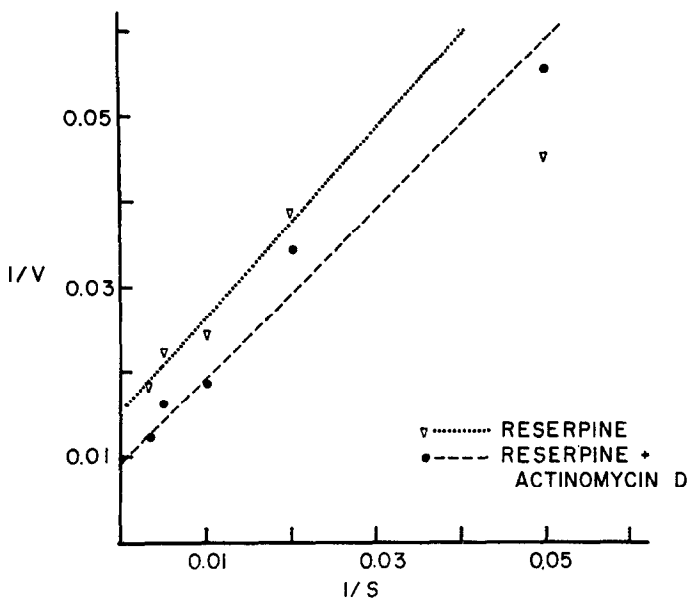

FIG. 6. Effect of reserpine and reserpine plus actinomycin D on uptake of NE by rat hearts. Hearts were perfused with *dl*-^{3}H-NE at the indicated concentrations (S = ng/ml as the free base). Initial velocity of uptake of NE (ng/g/min) was determined as described in the text. The animals which received reserpine alone were examined 4 days after administration of reserpine phosphate (2 mg/kg, i.p.). The hearts of animals which received actinomycin D and reserpine were examined 4 days after reserpine (2 mg/kg, i.p.) and 2 days after actinomycin D (100 μg/kg, i.p.). Kinetic parameters were computed as described under Methods, and statistical significance was assessed by using the calculated S.E.'s of the kinetic values and the appropriate degrees of freedom. The values of K_m (ng/ml) and V_{max} (ng/g/min) were not significantly different for the two groups.

	Reserpine	Reserpine + actinomycin D
K_m (ng/ml)	51 ± 19	110 ± 43
V_{max} (ng/g/min)	60 ± 19	106 ± 18

The effect of actinomycin D on adrenal medullary repletion of NE after reserpine

Although Kopin and Gordon[34] have shown that the normal rat heart obtains only about 20 per cent of its NE from the circulation, this figure might be higher in nerve terminals which are repleting their NE stores. Although levels of circulating NE were not measured in these experiments, presumably, a portion of such amine is of adrenal medullary origin. Thus these agents might be prolonging cardiac NE repletion after reserpine by delaying repletion of adrenal medullary stores, which normally are much more rapidly repleted than NE in other sites such as the heart.[3] The concentrations of NE in the adrenal medullae of rats which received actinomycin D (100 μg/kg, i.p.) 2 days after reserpine (2 mg/kg, i.p.) were not significantly different 4 days after reserpine from those of animals which received only reserpine (Table 3). Thus, there appears to be no effect of actinomycin D on the rate of repletion of adrenal medullary NE.

TABLE 3. EFFECT OF ACTINOMYCIN D ON REPLETION OF ADRENAL MEDULLARY NOREPINEPHRINE AFTER RESERPINE

Treatment	Days after drug	Adrenal NE (μg/mg protein ± S. E.)
None		1·18 ± 0·25
Reserpine*	2	0·06 ± 0·04
Reserpine*	4	1·30 ± 0·35
Reserpine*	4	1·18 ± 0·33
and actinomycin D†	2	

* 2 mg/kg, i.p.
† 100 μg/kg, i.p.

DISCUSSION

The most direct method to determine if the slow repletion of cardiac NE after its depletion by reserpine is due to the necessity for resynthesis of storage sites would be to determine the rate of synthesis of the storage sites. Potter and Axelrod[35,36] have shown that the vesicular structures obtained by differential or density gradient centrifugation of the rat ventricle are similar to those isolated from bovine splenic nerve.[37] Presumably, other types of binding sites in adrenergic neurons may exist, but these have yet to be described. Much work has been done to characterize the neuronal vesicles and those of the adrenal medulla. Both have been found to contain large amounts of phospholipid, RNA, protein, and ATP.[37,38] Presumably, the rate of synthesis of one of these components of the vesicles might be measured by determining the incorporation of suitable precursors into any one of these fractions. Attempts to determine the rate of incorporation of RNA or protein precursors into the vesicles of the HSSF have not been successful thus far.*

The work of Khan and Wilson[17] and of Wilson and Dove[39] has indicated only a very slow turnover of central nervous system subcellular structures which contain appreciable amounts of RNA and protein. They saw two different rates of decline in relative sp. act. of nonaqueous, nonlipid tritium in brain microsomes isolated from animals which had received $H_2{}^3O$ after conception. One component, representing 88 per cent

* Authors' unpublished observations.

of the microsomal fraction, had a half-life of 15 days, and a second small component had a half-life of 120 days. This same group has found similar disappearance curves for the mitochondrial components of other tissues. No quantitative data comparing metabolic activity of central nervous system tissue and peripheral nervous system tissue, such as the sympathetic nerve endings in the rat ventricle, are available. Therefore, it is possible that in the peripheral sympathetic neuron the rate of turnover of these microsomal-like neuronal vesicular structures is also very slow. Dahlstrom and Haggendahl[40] have calculated the average life-span of rat amine storage vesicles to be 35 days.

It is obvious that it was not possible to test the assumed capacity of the metabolic inhibitors utilized in this study to decrease the rate of turnover of NE storage sites in control animals, However, this conclusion does not rule out the possibility that the inhibitors used do further retard a slow turnover rate, or that possibly the turnover rate after reserpine is increased to restore the binding capacity and that this increased rate of turnover is decreased by administration of the inhibitors. For this reason it was deemed desirable to utilize several agents which have been shown to inhibit the synthesis of RNA, protein, or both in different tissues and possibly by different mechanisms.

Thioacetamide, in the dose chosen here, has been shown to inhibit the synthesis of rat hepatic microsomal RNA and protein.[41,42] Weisberger *et al.* have found the methylsulfonyl analog of chloramphenicol inhibits antibody synthesis in rabbit lymphatic tissue,* as does its parent compound.[43] These workers used a dose of 0·05 to 0·1 g/kg of the analog, half the dose used in the present study. It is now generally accepted that interaction of antinomycin D with DNA decreases the rate of RNA and protein synthesis.[44]

Although it was once thought that reserpine was rapidly metabolized, more recent work by Sheppard *et al.*[8] as well as by Maggiolo and Haley[9] has indicated that very small but detectable amounts of reserpine and its major metabolites are still present many days after a single dose of the drug. Thus it is possible that the persistence of reserpine is responsible for the prolonged depletion of catecholamines and serotonin. If this is the case, it is conceivable that, if the enzymes responsible for inactivating reserpine were inhibited, this could also cause a prolongation of reserpine-induced NE depletion. The inactivation of many drugs has been shown to occur in the microsomal fraction of the liver. As previously mentioned, reserpine is metabolized by the microsomal fraction of guinea pig livers.[31] Sladek and Mannering[30] have shown that prior administration of thioacetamide can decrease the rate of *N*-demethylation of ethylmorphine seen in rat liver microsomes. Thus, thioacetamide could be exerting its effect by interfering with the metabolism of reserpine. Since thioacetamide and chloramphenicol were both administered for several days before reserpine administration, perhaps both agents decrease the capacity of the liver microsomal drug-metabolizing enzyme system to inactivate reserpine.

The inhibitory effect of SKF 525-A on numerous reactions which take place in liver microsomes is now well known.[45, 46] Hence, the alteration by SKF 525-A of the repletion of cardiac NE after reserpine depletion was initially thought to be due primarily to inhibition of reserpine metabolism. Since the action of SKF 525-A on liver

* Personal communication.

microsomal enzymes is evident within hours,[47] prolonged administration prior to reserpine administration was not necessary. If the small amount of residual reserpine present 24 hr after its administration is responsible for the persistent NE depletion, and if the residual reserpine is metabolized predominantly in the liver, it should be possible to see a prolongation of NE depletion when SKF 525-A is given after reserpine. It must be pointed out, however, that numerous esterases may be able to hydrolyze the ester linkage of reserpine. Moreover, some extramicrosomal enzymes are inhibited by SKF 525-A, e.g. mouse serum procaine esterase.[48] In any event, this inhibitor, when given after reserpine and in the dosage employed, had no effect on the rate of disappearance of ^{3}H-reserpine from the liver, heart, or the entire body of the rat, even though it did retard the rate of NE repletion in myocardial nerve terminals. Thus, it would seem unlikely that the prolonged depletion of NE produced by this agent is due to an inhibition of reserpine metabolism. This is supported by the work of Sheppard and Tsien,[31] who found no correlation between the minimal effective dose of reserpine and the rate at which the methylreserpate-trimethoxybenzoic acid ester bond is hydrolyzed in different species. It is interesting that in our experiments, when SKF 525-A was administered before reserpine, no prolongation of NE repletion was observed. Since Neubert and Timmler[33] have demonstrated a diminished capacity of crude rat liver homogenates to incorporate ^{14}C-alanine into protein when exposed to SKF 525-A, it is possible that at the large doses of SKF 525-A employed after reserpine administration we again see a prolongation based on inhibition of resynthesis of storage sites.

Actinomycin D has also been observed to decrease the activity of the rat hepatic microsomal system.[49] Therefore, the effect of this inhibitor upon residual cardiac ^{3}H-reserpine concentration was also determined, but no increased accumulation of reserpine occurred with this inhibitor. Thus, the effects of actinomycin D on NE repletion after reserpine do not appear to be explained by an interference with the amine storage mechanism, nor by intereference with the disappearance of reserpine; hence, it may be acting by inhibiting the resynthesis of NE storage sites.

Since the neuronal vesicles contain protein as a structural component, presumably ribosomes would be required for their synthesis. However, no electron photomicrographic evidence of axonal ribosomes has ever been presented, even in regenerating nerve stumps, where it would seem they might be most readily found.[50] Thus, if new synthesis of storage sites is necessary, it would most probably occur in the perikaryon area. That this indeed may be true is further supported by the work of Dahlstrom *et al.*[51] In their study, a formaldehyde fluorescent stain technique was utilized to visualize NE and epinephrine (E) in the brain stem of the rat. They noticed that after the depletion of NE and E by reserpine, fluorescence due to these catecholamines first returned in the perinuclear areas, often within 24 hr. Only after 72 hr was fluorescence noted in the nerve terminals in the central nervous system. Dahlstrom[52] also noted that, when a peripheral nerve such as the sciatic or greater splanchnic nerve was constricted, an accumulation of amine granules occurred both above and below the constriction, the latter presumably being due to retrograde flow of more peripheral vesicles toward the site of constriction. If reserpine was given at the time of constriction, only the accumulation of vesicles above the constriction was evident, and this occurred only after there was a reappearance of catecholamines in the cell bodies. On the basis of these findings they have suggested that neuronal vesicles are formed in the cell body and

subsequently travel down the axon to the nerve terminal.[51] This is an extension of the axoplasm flow theory of Weiss and Hiscoe.[53]

If we assume that axoplasm flow soon transports the newly synthesized granule outside the cell body area and into the axon, this might explain the inability to detect catecholamine vesicles in nerve cell bodies of sympathetic ganglia by electron photomicroscopy or density gradient studies of ganglionic homogenates. Perhaps differences between the uptake and storage of NE in cell bodies and nerve endings of sympathetic neurones are related to a deficit in this storage form.[54]

Sartorelli and Magus[55] have shown that restoration of serotonin to normal levels in mouse kidneys after its depletion by 5 μmole/kg reserpine phosphate is delayed by subseqent administration of actinomycin D (0·1 μmole/kg). They interpreted this to indicate that DNA-directed RNA synthesis is a prerequisite for repletion of monoamine stores. In the present experiments it has been shown that these results also obtain for repletion of NE after reserpine depletion.

It was believed desirable to attempt to explain the present findings with actinomycin D and the other inhibitory agents by other mechanisms, apart from the more widely recognized effects already discussed. The possibility that these agents, as well as the others used in these studies, might be exerting their effects by decreasing dietary intake was controlled by the paired feeding technique. It is also possible that their effects on cardiac NE repletion are simply a secondary phenomena. Since up to 20 per cent of cardiac NE in normal rats may be derived from NE carried to the heart by the circulation, and since the adrenal medulla may contribute to circulating NE levels, a possible effect of actinomycin D on adrenal NE repletion after reserpine depletion was examined. Such an effect was not demonstrated.

It was also believed that these agents (thioacetamide, actinomycin D, the methylsulfonyl analog of chloramphenicol) could be altering the capacity of the nerve terminals to remove NE from perfusing solutions, as determined on the basis of calculated uptake kinetic constants. This would imply that no further alteration of the uptake process occurs with actinomycin D. Also, no differences were observed in the total retention or in the subcellular distribution of ^{3}H-NE 2 hr after i.v. administration in control and thioacetamide-treated animals. Therefore, it would seem unlikely that retention of ^{3}H-NE was affected by thioacetamide.

The possibility also was considered that the agents demonstrated in this study to be capable of affecting repletion of NE after reserpine administration might alter the rate of synthesis of NE within the nerve terminals. Actinomycin D was selected for further exploration of this possibility. No difference in the rate of ^{3}H-NE disappearance was observed between normal rats and those which had received actinomycin D 18 hr before ^{3}H-NE was administered. The failure of any of the agents used in these studies to alter normal catecholamine levels in the dosage regimens utilized might be expected if under normal conditions the neuronal vesicles are resynthesized very slowly.

The alternative explanations of the prolongation of reserpine depletion seen with these agents, i.e. alterations of amine storage mechanisms, appear to be unlikely explanations. Our results are believed to be compatible with the hypothesis that the restoration of normal amine storage mechanisms after reserpine administration depends upon the synthesis of new storage sites within the neuron.

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