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DOES TETRAHYDROPAPAVEROLINE CONTRIBUTE TO THE CARDIOVASCULAR ACTIONS OF DOPAMINE?

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Abstract—It has been previously shown *in vitro* that dopamine (DA) in the presence of monoamine oxidase condenses with its corresponding aldehyde (dihydroxyphenylacetaldehyde) to yield tetrahydropapaveroline (THP), an isoquinoline derivative structurally related to papaverine, which exhibits β-adrenergic activity. A sensitive method has been developed for the extraction and separation of dopamine-2-^{14}C (DA-2-^{14}C) and its metabolites by using thin-layer chromatography and liquid scintillation counting. At 2 and 5 min after the i.v. injection of 250 μg/kg of DA-2-^{14}C, samples of several guinea pig tissues were analyzed for THP-^{14}C. With the exception of the liver, no tissue contained any amount of THP greater than that seen in samples which served as controls for nonenzymatic conversion of DA to THP-like materials during the course of the extraction procedure. An explanation of the failure to detect significant amounts of THP *in vivo* was found when varying concentrations of DA were incubated with guinea pig liver mitochondria. When 1 mg/ml of DA-2-^{14}C was incubated, approximately 15 per cent of the radioactivity was converted to THP in 5 min, while the major metabolite was dihydroxyphenylacetic acid. Incubation *in vitro* of 0·24 μg/ml of DA-2-^{14}C, which more closely approximates the level of DA *in vivo* after the injection of the above dose, resulted in a conversion of only 3 per cent of the DA to THP. It was concluded that the cardiovascular responses seen with DA are not mediated by THP, since at most only trace amounts were detected. Based on the potency of THP, these amounts are considerably below the quantities of THP necessary to produce the depressor response.

THE INCUBATION of milligram quantities of dopamine (DA) with guinea pig liver monoamine oxidase preparations leads to the formation of dihydroxyphenylacetaldehyde, which condenses with unchanged DA to form tetrahydropapaveroline (THP).[1, 2] THP has been shown to be pharmacologically active and capable of stimulating β-adrenergic receptors.[2, 3] It has been suggested that THP may be active in mediating some of the cardiovascular actions of DA *in vivo*.[4, 5] If THP is to contribute significantly to the pharmacological actions of DA, one would expect to find in the tissues after the injection of DA amounts of THP which are commensurate with its pharmacological activity. In addition, the level of THP should be at its maximum at the height of the pharmacological response. In the present study, thin-layer chromatography (TLC) and autoradiography of butanol–heptane extracts of a number of guinea pig organs have been utilized to determine if these criteria are fulfilled after i.v. administration of 250 μg/kg of DA-2-^{14}C, which produces a

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vasodepressor response. The experiments show that only an insignificant amount of radioactivity is associated with carrier THP after the i.v. administration of DA-2-^{14}C, making it unlikely that THP contributes significantly to the cardiovascular actions of DA.

MATERIALS AND METHODS

Dopamine-2-^{14}C (21·8 mc/m-mole and 32 mc/m-mole) was purchased from Nuclear Chicago, and determined to be 95 per cent pure by TLC, autoradiography and counting of the radioactive areas. A radiochemical contaminant was found having an R_f value identical with that of THP. Attempts to remove this THP-like contaminant by TLC proved unsuccessful, because of further degradation of the DA during its elution from the chromatoplates. Any further attempts to purify the DA were therefore abandoned due to the small amount of this impurity (1–2 per cent, depending upon the lot). Dopamine HCl was obtained from the Nutritional Biochemical Co. Dihydroxyphenylacetic acid (DPA) was purchased from K & K Laboratories, Inc. THP was a gift from Dr. H. T. Openshaw, Wellcome Research Laboratories, Beckenham, Kent.

Acetone was repurified by distilling 1 l. over 1·5 g $KMnO_4$.[6] "Acid-acetone" was prepared just before use by shaking 100 ml acetone for 30 min with an excess of sodium metabisulfite. The resulting suspension was filtered twice and 1 ml of concentrated HCl was added after the second filtration. The solution occasionally had an opalescent appearance which did not affect the results. *n*-Butanol was redistilled and *n*-heptane was purified by distillation over H_2SO_4 (500:25; v/v). Nitrogen gas was purchased from the Matheson Co. under the name "Nitrogen prepurified".

Chromatography. Chromatography of DA and its metabolites was carried out by using the following solvent systems: solvent 1, water-saturated *n*-butanol : glacial acetic acid (6:1); solvent 2, *n*-butanol : formic acid : water (15:3:2).[1] Glass plates (20 × 20 cm) were coated with silica gel GF_{254}, 250 μ thick, or with cellulose MN300G, 250 μ thick, by using a Desaga adjustable spreader (Brinkmann). Slurries for the preparation of the plates were made by using 0·067 M phosphate buffer, pH 6·8, containing 1 % Na metabisulfite instead of water. Cellulose slurries were prepared by mixing 15 g MN300G with 80 ml of the buffer in a VirTis 45 mixer for 1 min. Silica slurries were prepared by shaking 25 g silica with 55 ml of the buffer for 1 min. After the slurries were spread, they were allowed to air dry for 20 min and were then dried for 15 min at 105°. The plates were stored in a desiccator over silica gel.

Extraction and analysis. Male guinea pigs weighing between 350 and 600 g (average, 465) were starved for 12–18 hr and then anesthetized with urethane, 1·6 g/kg, administered i.p. A tracheostomy was performed, followed by cannulation of the jugular vein, and heparin (200 units/kg) was injected. Blood pressure recordings from the carotid artery were made using an E and M model P-1000 pressure transducer and recorded on an E and M Physiograph. After the initial preparation and stabilization of the blood pressure, DA-2-^{14}C, 5 or 10 μc (approximately 250 μg/kg) in 0·2 ml of 0·01 N HCl, was injected i.v., washed in with 0·2 ml saline and the depressor response was monitored on the physiograph. The average fall in mean blood pressure in response to a 250 μg/kg dose of DA was 8·5 $\pm$ 2·0 mm Hg.

An incision was made from the jugular notch to the xiphoid process and, either at the peak of the depressor response to DA (1·5–2 min) or after the termination of the

response (5 min), the sternum was cut, the chest retracted, the heart rapidly removed, and the rest of the tissues under study were removed, trimmed of excess fascia, washed in ice-cold saline, blotted and frozen in liquid nitrogen. Approximately 3 min were needed to complete the dissection.

A sample of blood (usually 0·5 ml) was removed from the thorax after removal of the heart and centrifuged at 4° for 10 min at 2000 rpm (head No. 269) in an International refrigerated centrifuge, model Pr-1. The sample of plasma was removed and the red blood cells were resuspended in 0·5 ml saline and centrifuged for an additional 10 min. The saline layer was combined with the plasma sample and carried through the extraction while the red blood cells were discarded.

A modified acid-acetone extraction was used to isolate DA and its metabolites from the tissues for separation by TLC.[6] A control sample containing added DA-2-^{14}C and approximately 0·5 g kidney from another guinea pig was used to check for recovery and nonenzymatic conversion of DA to THP-like materials during the extraction procedure. The frozen tissues were crushed in a stainless steel percussion mortar under liquid nitrogen and the resulting tablets[7] were homogenized at 4° in a Potter–Elvehjem homogenizer containing 5 ml of ice-cold acid-acetone and 10 μg of carrier DA and THP. The homogenizer was washed once with 2 ml and then with 1 ml acid-acetone and the washings were combined with the original homogenate and transferred to a 10-ml polyallomar (Spinco) centrifuge tube. The tube was flushed with nitrogen, capped and kept in ice for one-half hr. Ninety-five per cent of the tissue radioactivity was extracted into the acid-acetone by this procedure. All proceeding steps were carried out at 4° except shakings and each step was performed under a nitrogen atmosphere.

The homogenate was centrifuged for 10 min at 20,000 rpm in a Spinco model L ultracentrifuge with a type 50 rotor. The supernatant was brought to a final volume of 10 ml with acid-acetone and a 0·5-ml or 1-ml aliquot was taken for radioassay. The supernatant was transferred to a 25-ml glass-stoppered shaker bottle and evaporated under nitrogen at approximately 55°. The solution of acid-acetone was evaporated to near dryness (approximately 0·01 ml). Five ml of ice-cold *n*-butanol saturated with 0·01 N HCl containing 0·01 % EDTA was added to the shaker bottle and shaken for 3 min.

The *n*-butanol layer was shaken for an additional 3 min against 10 ml *n*-heptane and 1 ml of 0·01 N HCl containing 0·01 % EDTA. The addition of the 10 ml of *n*-heptane partitioned the DA and THP out of the *n*-butanol layer and into the 0·01 N HCl phase. After shaking, the bottles were centrifuged for 5 min at 2000 rpm to separate the layers. The *n*-butanol–heptane layer was carefully aspirated off and the remaining HCl layer was shaken with an additional 5 ml of cold *n*-heptane for 3 min. After shaking, the mixture was centrifuged as described above, and the heptane layer was removed by aspiration. Occasionally, an emulsion formed at the interface between the HCl and heptane layers. The emulsion was broken by removing the excess heptane, freezing the sample in dry ice, thawing, centrifuging and repeating if necessary. The remaining HCl layer was removed and the bottle washed with an additional 1 ml of 0·01 N HCl (no EDTA), combined with the original HCl layer and transferred to an Aminco 5-ml freeze-drying tube and dried on an Aminco lyophilizer.

Reconstitution was achieved by adding a minimum of 0·25 ml acetone : 0·01 N HCl (5:1), capping the tube and carefully swirling the solvent around the tube for

several minutes with a Vortex mixer. After reconsitution, the samples were centrifuged as described above for the shaker bottles. Routinely, three chromatoplates (2 silica and 1 cellulose) of each reconstituted extract were prepared by spotting 50-μl aliquots onto silica and cellulose plates under nitrogen. The plates were developed with solvent 1 or 2. The walls of the developing chamber were lined with solvent-saturated filter paper and development under nitrogen was allowed to proceed for approximately 11 cm.

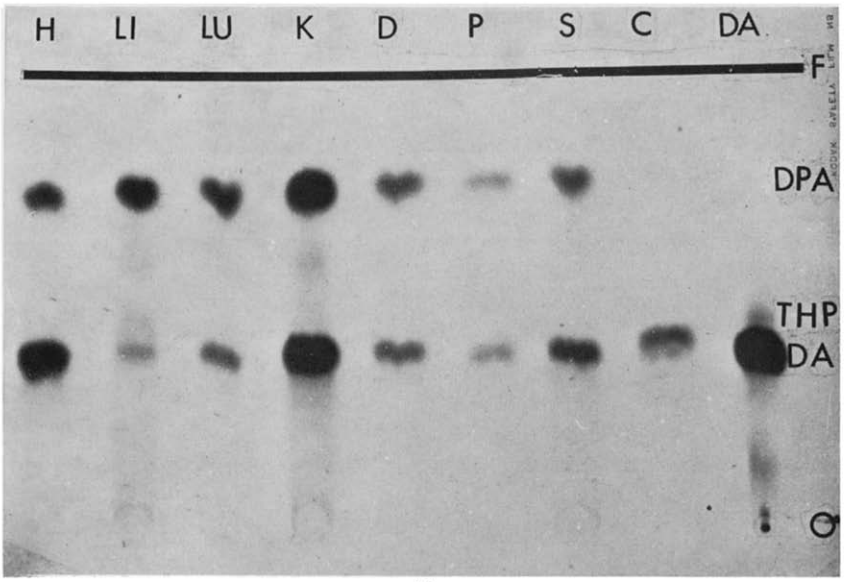
Autoradiograms were made of the air-dried chromatoplates with Kodak no-screen X ray film with the most common exposure time being 1 week. Carrier DA and THP on the dried plates were visualized by spraying with iodine spray (0·5%, w/v, iodine in chloroform). The areas corresponding to the spots on the autoradiogram and the carrier spots were scraped into counting bottles containing 0·1 ml of 0·01 N HCl and 10 ml phosphor solution[8] and shaken for 1 hr to insure complete elution from the adsorbant. The samples were counted in a Packard Tricarb liquid scintillation spectrometer with an average counting efficiency of 70–75 per cent based on internal standardization with toluene-^{14}C. Determinations agreed between plates within 10 per cent. Spots on the autoradiogram, having an approximate area of 2 cm^2 and containing 200 cpm above background, or slightly less, could be routinely detected with an exposure period of 1 week. With the sp. act. used in these experiments, this represents 1·5 to 3 ng DA or THP.

The recovery of authentic DA-2-^{14}C in control samples was approximately 85 per cent. The recovery of THP as determined by fluorescence assay[2] was the same as that for DA.

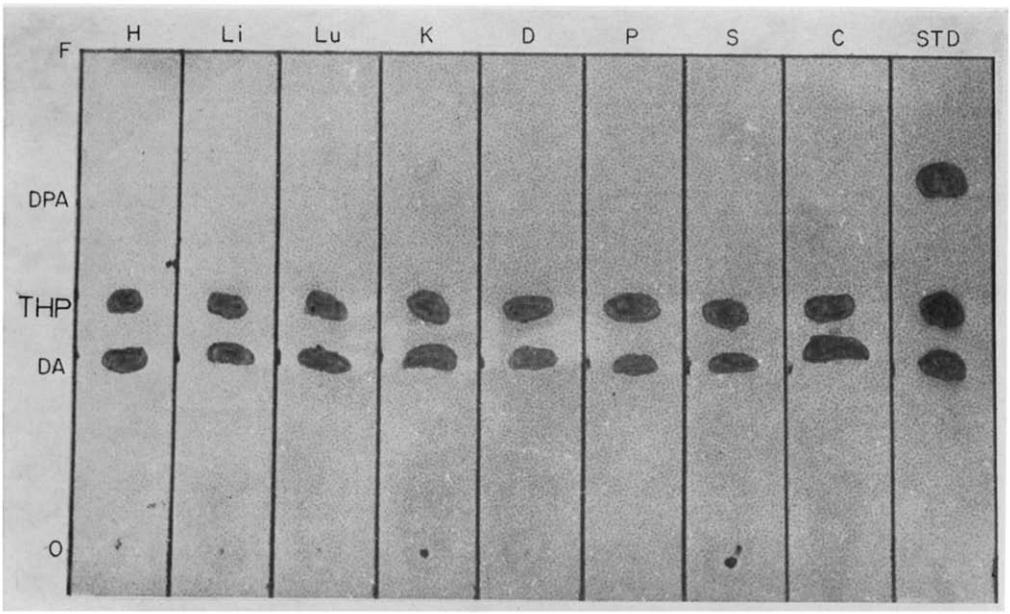
RESULTS AND DISCUSSION

Samples of the heart, liver, lungs, kidneys, spleen and plasma were investigated for the presence of ^{14}C-labeled materials associated with THP carrier after the intravenous administration of DA-2-^{14}C. Four guinea pigs were sacrificed at the peak of the depressor response (1·5 to 2·0 min after injection) and 3 guinea pigs were sacrificed at the end of the response (5 min after injection).

Two well defined spots were always observed on autoradiograms of silica gel TLC's of extracts of all tissues taken from guinea pigs at the peak of the depressor response and after its termination (Fig. 1 (a)). These spots corresponded in position with DA and its acid metabolites, dihydroxyphenylacetic acid (DPA) and homovanillic acid (HVA), which migrate as a single spot in this system. Depending on the tissue, between 10 and 44 per cent of the total radioactivity was associated with the DA carrier spot. The metabolites represented between 50 and 75 per cent of the total radioactivity of which 5–15 per cent was HVA. The HVA determinations were made on cellulose plates which separate it from DPA by using either solvent system. The corresponding chromatogram shows the presence of carrier DA and THP in all the tissues and control samples (Fig. 1 (b)). In this experiment, only in the liver and possibly in the kidney is there even minimal autoradiographic evidence of radioactivity associated with THP carrier. Radioactivity was also associated with an area just below the DPA spot in the liver and kidney which was not further characterized. Other possibly important metabolites of DA, norepinephrine and 3-methoxydopamine, did not migrate with THP in any of the solvent systems. The mobility of 3,4-dihydroxyphenylethanol was not determined because of its commercial unavailability. Although



(a)



(b)

FIG. 1 (a). Autoradiogram of a phosphate-buffered silica plate (solvent 2) showing radioactive metabolites in organs of a guinea pig injected with 20 μc/kg (250 μg/kg) DA-2-^{14}C and sacrificed at the peak of the depressor response. O = origin, F = solvent front, H = heart, LI = liver, LU = lung, K = kidney, D = duodenum, P = plasma, S = spleen, C = control (containing inactivated kidney and DA-2-^{14}C), DA = aliquot of original DA solution for injection, DPA = DPA + HVA. (b). The chromatogram of the autoradiogram shown in Fig. (a); STD = 5 μg each of DA-2-^{14}C, THP and DPA. Sprayed with I_2 in $CHCl_3$ (0·5 %, w/v). The carrier spots on the photograph of the chromatogram have been darkened for better photographic contrast.

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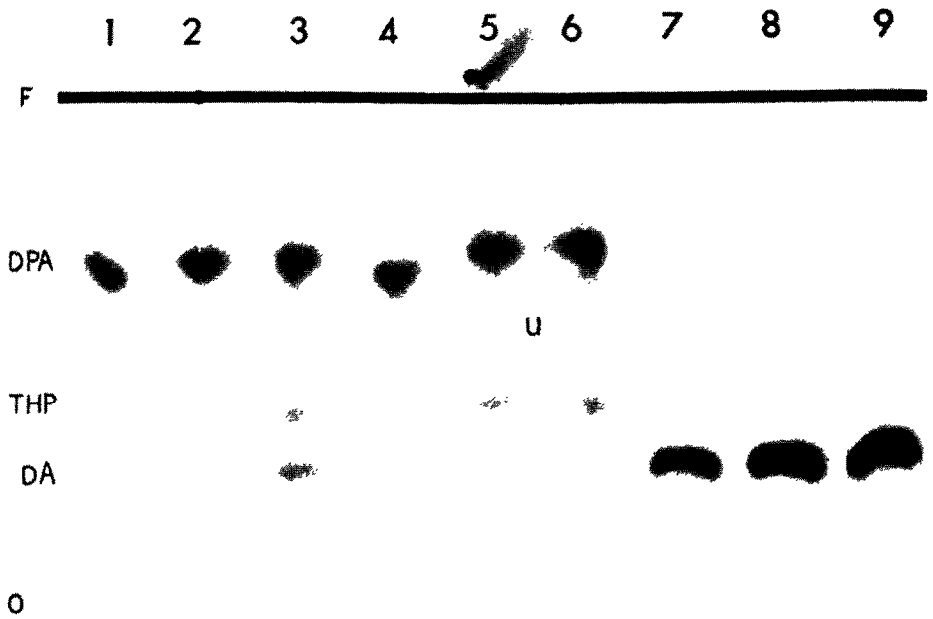

FIG. 2. An autoradiogram of the liver incubates chromatographed on a phosphate-buffered silica plate (solvent 1). 1 – 0·24 μg/ml concentration of DA incubated for 5 min; 2 and 3 – 1 mg/ml concentrations of DA incubated for 5 min; 4 – 0·24 μg/ml concentration of DA incubated for 10 min; 5 and 6 – 1 mg/ml concentrations of DA: incubated for 10 min; 7, 8 and 9 – boiled mitochondria controls. O – origin; F – solvent front; U – unknown, which may, however, be a tail of DPA.

this deaminated, reduced derivative of DA has been found in the urine of pyrogallol-treated rats after DA administration, it was not detected in the urine of normal rats.[9]

The identification of radioactivity associated with the THP carrier on the chromatoplate as THP formed *in vivo* must be evaluated in light of the occurrence of a THP-like contaminant of the ^{14}C-labeled DA used in this study, which could not be removed by the purification procedures employed. In order to account for the impurity as well as for its possible formation during the extraction procedure, an aliquot of the original DA solution was added to a sample of kidney tissue not previously exposed to any radioactivity and was carried through the extraction procedure. These experiments on the recovery of authentic DA showed that samples which had very small amounts of tissue, such as plasma, contained a radioactive material associated with carrier THP (8 per cent of the total radioactivity) which was consistently greater than that found in the other control samples (2–5 per cent) containing larger amounts of tissue. Because of the uncertainty of the tissue distribution and recovery of the THP-like contaminant present in the original DA solution, a maximum estimate of the THP levels *in vivo*, obtained by not subtracting the amounts of THP-like material in the control, is presented.

Exclusive of the plasma, at the peak of the depressor response, from 0·8 to 2·2 per cent of the total radioactivity in all tissues investigated was associated with THP carrier spots, with the liver showing the greatest percentage. The kidney, however, exhibited the largest amount of radioactivity associated with the carrier spot which was equivalent to 0·074 μg THP/g. All the other tissues displayed radioactivity equivalent to 0·01 to 0·02 μg THP/g. After the termination of the depressor response, there was no significant difference in the levels of radioactivity associated with the carrier THP spots. At the peak of the depressor response, approximately 8 per cent of the radioactivity in the plasma was associated with carrier THP, which was equivalent to 0·03 μg THP/g, and after the termination of the response these values did not change significantly.

When the amounts of radioactivity associated with the THP carrier spots are corrected for the THP-like material found in the tissue and plasma control samples, then amounts of THP greater than the control percentage were found only in the liver in 1 out of 4 animals at the 2-min interval (0·5 per cent) and in the livers in 2 out of 3 animals at the 5-min time period (1·6 and 1·0 per cent). Further characterization of these very small amounts of radioactivity as authentic THP was not pursued, since the maximum estimates of THP levels are far too low to have it serve as an active metabolite of DA. This conclusion follows from the fact that THP is approximately 10 times less potent than DA,[2] and even the maximum estimates of THP levels in the various tissues are 5–60 times less than the levels of DA and its acid oxidation product, DPA.

It has been shown that liver mitochondria produce significant amounts of THP from DA *in vitro*, which is in contrast to the results of the present experiments *in vivo*. An experiment was therefore conducted with liver mitochondria to determine the possible reasons for this discrepancy. After i.v. injection of 250 μg/kg of DA we found the levels of DA in the guinea pig liver to be of the order of 1 μg/g wet wt. or less. Since the concentration of DA might be a determinant in the amount of THP formed, guinea pig liver mitochondria were incubated with final concentrations of 0·24 μg/ml (0·13 μg/g, wet weight) and 1·0 mg/ml (0·56 mg/g, wet wt). The former concentration

(0·13 μg/g) more closely approximates that found in the liver 2 min after the injection of DA, while the latter concentration (0·56 mg/g) corresponds to that used by Holtz *et al.*[1]

Fig. 2 shows the autoradiogram of one of the two silica plates used in this experiment. In the boiled control samples (7, 8 and 9), radioactivity is associated only with DA, indicating the absence of nonenzymatic conversion during the incubation. The flasks (2 and 3) which contained 1 mg/ml of DA incubated for 5 min showed radioactivity corresponding to DA, THP and DPA, while those incubated for 10 min (5 and 6) showed THP and DPA, but no DA. It is presumed that no HVA was present in the DPA spot, since catechol-*O*-methyl transferase activity is in the supernatant fraction. On the other hand, only DPA was detected on the autoradiogram when 0·24 μg/ml of DA was incubated for 5 or 10 min (1 and 4).

Table 1 shows the quantitative results of this experiment obtained by scraping and counting of the carrier spots. The percentages of THP formed (approximately 3 per

TABLE 1. EFFECT OF DA CONCENTRATION ON THP FORMATION IN GUINEA PIG LIVER MITOCHONDRIA *in vitro**

Flask No.	Incubation time (min)	Concn of DA in flask	DA† (%)	THP† (%)	DPA† (%)
1	5	0·24 μg/ml	2·1	3·2	85·7
2	5	1 mg/ml	12·9	14·4	50·4
3	5	1 mg/ml	18·0	13·5	48·2
4	10	0·24 μg/ml	3·0	3·2	84·5
5	10	1 mg/ml	4·3	17·3	51·2
6	10	1 mg/ml	3·9	16·2	51·2
7‡	5	0·24 μg/ml	97·1	0·7	0
8‡	5	1 mg/ml	96·6	0·9	0
9‡	5	1 mg/ml	95·7	1·5	0

* Guinea pig liver mitochondria were prepared and incubated as described by Holtz *et al.*[1] Flasks were equilibrated for 5 min and the reaction was initiated by addition of DA from the side arm. All samples were incubated at 37° under oxygen. Reactions were terminated by the addition of 2·5 ml of ice-cold acid-acetone. Ten μg of carrier THP was added to all the flasks and 20 μg of carrier DA was added just to the flasks containing the lower concentration of DA, and after remaining in ice for one-half hr the incubates were spun for 20 min at 20,000 rpm in a Spinco model L ultracentrifuge. Fifty-μl samples of the supernatants were spotted on two phosphate-buffered silica plates and chromatographed by using solvent systems 1 and 2.

† The percentages represent the averages of two silica plates and were calculated as percentages of the total amount of radioactivity (dpm) in each column. The percentages do not add up to 100 because the radioactivity at the origin and in the zone between THP and DPA, which was not characterized, was also scraped and counted.

‡ Control flasks containing boiled mitochondria.

cent) during incubation of a microgram quantity of DA (flasks 1 and 4) agree with the results of the study *in vivo* with DA-2-^{14}C where a maximum of approximately 2·0 per cent of the total radioactivity was associated with THP carrier. At the end of these time intervals the amount of DA remaining is only 2–3 per cent of the total and approximately 85 per cent of it has been converted to DPA by the action of monoamine oxidase. This rapid conversion of DA to DPA agrees with what has been found

in vivo. The flasks (2, 3, 5 and 6) that contained the milligram concentration of DA formed 5 times the amount of THP on a percentage basis (approximately 15 per cent) after 5 and 10 min of incubation when compared to the incubates (1 and 4) containing the microgram concentration. During the interval from 5 to 10 min, the amount of THP increased only 2–3 per cent. The amount of DA present in the milligram concentration incubates (2 and 3) at the end of 5 min was approximately 7 times that present at the same time interval for the microgram concentration (flask 1). The concentration of DPA (50 per cent) in the 10-min flasks (5 and 6) containing the milligram concentration of DA may be somewhat below what would be expected based on the continued metabolism of DA. This is probably accounted for by what appeared to be an unidentified spot corresponding to area U (just below DPA) on the autoradiogram in columns 5 and 6. This may in fact be a tail of DPA which was caused by overloading of the plate. Since the true identification is uncertain, it was not considered as DPA, although it makes up approximately 20 per cent of the radioactivity on the column.

The results of the study *in vitro* allow us to formulate the following conclusions regarding the conditions necessary for the formation of THP in more than trace amounts. Only when 1 mg/ml of DA was incubated with the liver monoamine oxidase preparation were appreciable amounts of THP detected. Thus the significant formation of THP *in vitro* requires a high concentration of DA in the presence of monoamine oxidase activity. In this situation the concentration of DA exceeds the amount that is metabolized by monoamine oxidase within a short period of time. Consequently there is a large excess of DA present to condense with the aldehyde.

The failure to observe significant formation of THP when 0·24 μg/ml of DA was incubated with the monoamine oxidase preparations most likely results from the rapid oxidation of DA so that there is not an appreciable concentration of DA remaining to condense with the aldehyde. The results obtained with the incubation of the microgram quantity accounts for the fact that, at best, negligible amounts of THP are found *in vivo* after the injection of the 250 μg/kg dose of DA.

The results of these experiments therefore make it improbable that THP is responsible for the β-adrenergic cardiovascular effects of i.v. injected DA. The high concentrations of DA needed for the significant formation of THP *in vivo* are not achieved by the maximum pharmacological dose of DA which produces a depressor response.

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