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Demonstration of 3,4-dihydroxy[^{14}C]benzoic acid and [^{14}C]vanillic acid after administration of [^{14}C]noradrenaline in the rat

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Rats were injected with 40 μCi (±)-[1-^{3}H]noradrenaline and 10 μCi ±-[1-^{14}C]noradrenaline. Three fractions with a decreased ^{3}H:^{14}C ratio were isolated from the urine by a combined alumina adsorption-ethyl acetate extraction procedure. Two of the fractions were identified as [^{14}C]vanillic acid and 3,4-dihydroxy[^{14}C]benzoic acid, respectively. Vanillic acid represented between 1·5 and 3·0% of the total [^{14}C]activity excreted within 24 h and the contribution of dihydroxybenzoic acid was 0·2–0·5%. The third fraction with a decreased ^{3}H:^{14}C ratio has not been identified and represented about 2% of the total [^{14}C]activity excreted within 24 h. After monoamine oxidase blockade with 100 mg/kg of iproniazid, the excretion of vanillic acid, 3,4-dihydroxybenzoic acid and the unknown fraction was greatly diminished. The probability that these three substances represent those metabolites arising simultaneously with the formation of tritium water from (±)-[1-^{3}H]noradrenaline is discussed.

The metabolic fate of catecholamines in animals and man has been extensively studied ever since radioactively-labelled compounds became available. After administration of noradrenaline, only a small fraction leaves the body unchanged, most being metabolized before excretion. The two enzymes responsible for its metabolic degradation *in vivo* are catechol-*O*-methyltransferase (COMT) and monoamine oxidase (MAO). After administration of (±)-noradrenaline labelled with tritium on the C-1 (or β–C) {2-amino-1-(3,4-dihydroxyphenyl)-[1-^{3}H]ethanol; ^{3}H-NA}, tritium water (THO) was identified in the plasma of man (Gitlow, Mendlowitz & others, 1964), cats (Suko, Linet & Hertting, 1967) and rats (Pichler, Suko & Hertting, 1968). However, neither the action of COMT nor MAO can explain the loss of the tritium label at this position.

There are several possible transformations of ^{3}H-NA that would lead to the loss of the tritium label. (i) An unspecific exchange of labile tritium. (ii) The oxidation of noradrenaline to noradrenochrome and noradrenolutine, a mechanism that has been repeatedly discussed (Kety, 1959); here rearrangement of the noradrenochrome molecule to noradrenolutine would also cause loss of the tritium atom. Enzymic formation of adrenochrome in tissue homogenates has, indeed, been shown to occur by Axelrod (1964). (iii) The formation of vanillic acid (VA), a minor metabolic pathway demonstrated in man for adrenaline by Sandler, Ruthven & Wood (1964) and Goodall & Alton (1965), and for noradrenaline by Rosen & Goodall (1962) and Sandler & others (1964). The corresponding catechol compound 3,4-dihydroxybenzoic acid (DHBA) was found in the urine of man after administration of 3,4-dihydroxymandelic acid (DHMA) by Goodall & Alton (1969).

The double label technique was implemented, using a mixture of (±)-1-^{3}H-NA and (±)-1-^{14}C-NA in a given ratio, to investigate which of these transformations

accompanies the formation of THO in the body. Various analytical techniques were used to isolate the metabolites of noradrenaline, those fractions showing a decreased $^{3}H:^{14}C$ ratio must contain the metabolites in question. This procedure, moreover, simplifies further purification and identification of these compounds.

METHODS

Administration of drugs and urine collection

Male Wistar rats (250–350 g) were intravenously injected with a mixture of 40 μCi ^{3}H-NA (NENCO, specific activity 8·76 Ci/mmol) and 10 μCi ^{14}C-NA (CEA, specific activity 20·5 mCi/mmol). In some rats, iproniazid (100 mg/kg base) was injected intraperitoneally 16 h before the experiment to block MAO activity. Urine was collected in glass vials containing 0·1 ml of a mixture of 70% perchloric acid, 1% ascorbic acid and 5% EDTA, 2:2:1 by volume. The collection periods were 0–4, 4–8 and 8–24 h. The urine volume was 2–4 ml per sample. The collected urine was centrifuged and 50 μg of each of the following compounds added as carriers: DHBA, DHMA, 3,4-dihydroxyphenylglycol (DHPG), VA, vanillyl-mandelic acid (VMA), 4-hydroxy-3-methoxyphenylglycol (MHPG). The samples were then stored at −18°. Aliquots of urine were hydrolysed before the isolation procedure either enzymically with Glusulase (Endo) or by heating at pH 1 at 100° for 15 min.

Isolation procedure

The samples were recentrifuged after being thawed and the [^{3}H] and [^{14}C] content determined in an aliquot of the supernatant using a Packard liquid scintillation spectrometer (Okita, Kabara & others, 1957). Ascorbic acid (0·5 ml, 2%) and EDTA (0·1 ml, 5%) were then added, the samples brought to pH 5·5 and 0·7 g Al_2O_3 (Woelm, neutral) added with continuous stirring. The pH was then adjusted to 8·5. The alumina was transferred to columns, washed and eluted with 0·25N HCl. Eluates and effluents plus washings were then brought to pH 1 and processed separately as shown in Fig. 1. Aliquots of the final ethyl acetate samples were evaporated and taken up into 0·2 ml of ethyl acetate for paper chromatography. Other aliquots of the ethyl acetate extracts were evaporated directly in counting vials, 2 ml of methanol and 10 ml of toluol scintillator added and the radioactivity measured. To determine the extent of contamination of the isolated metabolite fractions by unaltered noradrenaline or by impurities present in the administered mixture of ^{3}H- and ^{14}C-NA, a diluted (1:10) solution of this mixture was subjected to the whole isolation procedure and the appropriate corrections made.

Each step of the isolation procedure was followed by paper chromatographic analysis in isopropanol–ammonia–water (8:1:1) and n-butanol–acetic acid–water (60:15:25) for effluents and n-butanol–acetic acid–water (60:15:25) and n-butanol saturated with N HCl for eluates. Distribution of radioactivity on the chromatograms was determined using a 4π-chromatogram scanner; the unlabelled reference compounds were developed by spraying the chromatograms with diazotized *p*-nitroaniline to detect *O*-methylated products and with the reagent of Goldenberg, Faber & others (1949) for catechol compounds.

In several experiments the chromatograms were cut in 1 cm strips, the activity eluted and the $^{3}H:^{14}C$ ratio of the eluates determined, whilst in other experiments the eluates of the radioactive peaks were further purified by rechromatography.

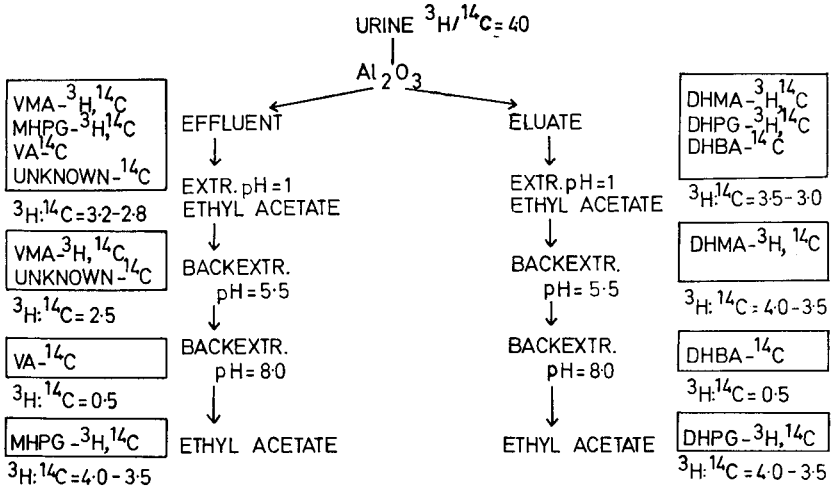

FIG. 1. Isolation procedure for various noradrenaline metabolites from rat urine. Rats were injected with 40 μCi (±)-1-^{3}H-NA and 10 μCi (±)-1-^{14}C-NA. The urine was collected and applied to alumina columns. Eluates and effluents were extracted into ethyl acetate at pH 1. The organic phases were back-extracted first into McIlvaine citrate-phosphate buffer at pH 5·5. The organic layer of the alumina effluents was then extracted with borate-HCl buffer (0·2M) at pH. 8·0, whilst the organic layer of the eluates was extracted with tris-HCl buffer (0·2M) at pH 8·0. The buffer extracts were adjusted to pH 1 and re-extracted with ethyl acetate. The metabolites found in the buffer extracts and in the remaining ethyl acetate phases are shown and the ^{3}H: ^{14}C ratios of the various fractions given.

Identification of metabolites with a low ^{3}H:^{14}C ratio

Information about the identity of metabolites with a low ^{3}H:^{14}C ratio was initially obtained from the coincidence of the R_F values of the radioactive peaks and of the reference compounds in various chromatographic systems. Furthermore, after rechromatography, for purification, and elution, the radioactive and reference compounds behaved identically on alumina columns and on extraction as detailed in Fig. 1. For more precise identification of the radioactive peak that possessed a R_F value similar to VA, the peak was rechromatographed, eluted and chromatographed on a Dowex-1-acetate column (9 × 500 mm) according to Weise, McDonald & LaBrosse (1961), after the addition of VA and VMA as carriers. The column was eluted with a convex concentration gradient (1·5–6·0M) of ammonium formate at pH 8·0 (Fig. 2) and the eluate collected in 7 ml fractions. The VMA and VA content in the fractions was measured directly by absorption at 279 nm, whilst the radioactivity was measured in ethyl acetate extracts (pH 1).

The radioactive peak corresponding to DHBA was eluted from the paper chromatogram and *O*-methylated enzymically (Axelrod & Tomchick, 1958). After the mixture had been incubated for 2 h at 37° it was extracted at pH 1 with ethyl acetate and the identity of the *O*-methylated product formed was established by column and paper chromatography.

Recovery of vanillic acid

DHBA was *O*-methylated enzymically using methyl-[^{14}C]-*S*-adenosylmethionine by the procedure of Axelrod & Tomchick (1958). The ^{14}C-VA formed was isolated by extraction into ethyl acetate at pH 1 and further purified by back-extraction (Fig. 1) and paper chromatography. This purified ^{14}C-VA was then added to rat

urine and the whole isolation procedure applied to determine the recovery of VA; 82% of the radioactivity added to the urine was recovered in the fraction of the alumina effluent isolated by back-extraction at pH 8·0.

RESULTS

The $^{3}H:^{14}C$ ratios of the various fractions isolated are shown in Fig. 1. The $^{3}H:^{14}C$ ratio has already decreased in the organic phase obtained after extraction of the alumina effluents and eluates into ethyl acetate at pH 1.

Paper chromatography of the fraction isolated by back-extraction at pH 5·5 of the eluate extract showed one radioactive peak identical with DHMA, the $^{3}H:^{14}C$ ratio of this fraction ranging from 4 to 3·5. Back-extraction at pH 8·0 isolated a chromatographically-uniform compound which corresponded to DHBA. The $^{3}H:^{14}C$ ratio of this fraction was 0·5. Rechromatography and elution of the DHBA peak led to a further great decrease in the $^{3}H:^{14}C$ ratio to 0·01. Enzymic *O*-methylation of the eluted peak resulted in the formation of VA. The radioactivity remaining in the organic phase proved to be ascribable to DHPG, the $^{3}H:^{14}C$ ratio lying between 4 and 3·5. There was no indication of the presence of 3,4-dihydroxy[^{14}C]benzylalcohol in this fraction.

The $^{3}H:^{14}C$ ratio of the fraction isolated by back-extraction at pH 5·5 of the effluent extract was decreased to 2·5. Paper chromatography revealed the existence of two peaks. One peak had the same R_F value as the carrier VMA and possessed a $^{3}H:^{14}C$ ratio of 3·5, the second peak was more polar than VMA and had a R_F of 0·61 in the butanol–acetic acid–water system and a R_F of 0·13 in the isopropanol–ammonia–water system. The $^{3}H:^{14}C$ ratio of this latter peak was 0·5. The metabolite responsible for this activity has not yet been identified; it accounted for approximately 2% of the excreted ^{14}C total activity.

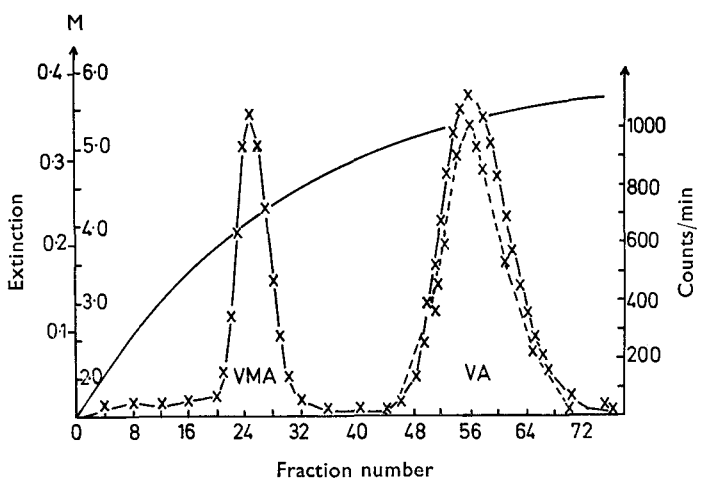

FIG. 2. Anion exchange chromatography of [^{14}C]vanillic acid isolated from the urine of rats that had received ^{3}H-NA and ^{14}C-NA. ^{14}C-VA purified from rat urine to which carrier VA and VMA (1 mg each) had been added was applied to a 500 × 9 mm Dowex-1-acetate column and eluted in fractions of 7 ml with a convex concentration gradient of 1·5–6·0M ammonium formate. The VMA and VA content was measured by absorption at 279 nm. For determination of radioactivity the fractions were acidified, extracted into ethyl acetate and the extracts measured in a liquid scintillation spectrometer. Left outer ordinate: extinction of added carrier VMA and VA, left inner ordinate: molarity of ammonium formate gradient, right ordinate: radioactivity as counts/min. Solid line represents extinction at 279nm, broken line represents radioactivity.

The fraction isolated by back-extraction at PH 8·0 of the effluent extract had a $^{3}H:^{14}C$ ratio of 0·5 and contained one radioactive peak corresponding to VA. Further chromatographic purification resulted in a $^{3}H:^{14}C$ ratio of 0·004. The pattern of the radioactivity in this fraction after elution from a Dowex-1-acetate column is shown in Fig. 2. It can be seen that the radioactivity leaves the column in the same pattern as the added carrier VA.

MHPG was found in the remaining ethyl acetate fraction, the $^{3}H:^{14}C$ ratio of which was 3·5. Vanillyl alcohol added as a carrier appeared in this fraction of our isolation procedure, but there was no radioactive peak with identical R_F values demonstrable on the paper chromatograms of this fraction.

Of the radioactivity administered, 60–70% was excreted in the urine within 24 h. After the appropriate corrections for recoveries had been made, ^{14}C-VA represented 1·5–3% and ^{14}C-DHBA 0·2–0·5% of the total [^{14}C]activity excreted. Neither enzymic nor acid hydrolysis of the urine increased the yield of ^{14}C-VA and ^{14}C-DHBA.

Blockade of MAO by iproniazid caused a decrease in the excretion of the total activity in the urine to about 50% of the administered radioactivity during the 24 h period. In these animals, ^{14}C-VA represented 0·3% of the excreted total [^{14}C]-activity, whereas ^{14}C-DHBA virtually disappeared. The unknown metabolite contained in the fraction isolated by back-extraction at pH 5·5 from the alumina effluent also disappeared after MAO-blockade.

DISCUSSION

Using the double label method, three fractions with a decreased $^{3}H:^{14}C$ ratio were isolated from the urine of rats that had received ^{3}H-NA and ^{14}C-NA. Two of these fractions were identified as VA and DHBA. The metabolite responsible for the third [^{14}C]-enriched fraction is as yet unidentified. The alcohols corresponding to VA and DHBA were not found in the urine of these animals.

During the 24 h collection period the sum of the metabolites without tritium label was between 4 and 5% of the excreted total [^{14}C] activity. In man, Rosen & Goodall (1962) found that 2·6% of the total activity, excreted within 24 h after the infusion of (±)-1-^{14}C-NA was vanillic acid. Our VA values in the rat of 1·5–3·0% of the excreted [^{14}C] activity are in the same range.

Dirscherl, Thomas & Schriefers (1962) observed the formation of VA from VMA in the perfused rat liver. The formation of DHBA in liver slices and homogenates incubated with DHMA was described by Thomas (1966). Goodall & Alton (1969) infused 1-^{14}C-DHMA in man and recovered large amounts of DHBA (7·7% of the total infused radioactivity) and smaller amounts of VA (2·0%) from the urine. These experiments indicate that the deaminated catecholamine metabolites serve as substrates for the formation of DHBA and VA. The considerable decrease in the formation of DHBA and VA after MAO blockade found in our experiments seems to indicate that deamination must take place before the removal of the 2-C-atom. Since in the rat the main deaminated metabolites of noradrenaline are the alcohols, MHPG and DHPG rather than the acids, VMA and DHMA, it can be assumed that the alcohols can also serve as precursors for DHBA and VA synthesis. If an isotope effect were involved in the formation of ^{14}C-VA and ^{14}C-DHBA, this should be reflected in an increase in the $^{3}H:^{14}C$ ratio of the precursors, VMA and DHMA. Since the $^{3}H:^{14}C$ ratios of the VMA and DHMA, as well as MHPG and DHPG fractions were close to 4, an isotope effect can be excluded.

In the rat, about 10% of administered $^{3}H:^{14}C$-NA undergoes a transformation in the body, which leads to a loss of the tritium atom attached to the side chain of the noradrenaline molecule (Pichler & others, 1968). About 50% of the THO formed from the ^{3}H-NA can be accounted for by the sum of VA, DHBA and the unknown metabolite isolated. Using our experimental procedure, noradrenochrome and noradrenolutine would be expected to appear in the alumina eluate, but there was no paper chromatographic indication for their presence. This is in accordance with the fact that the catecholamines are specifically stored within the sympathetic nerves and are, therefore, not accessible to degradation by catechol oxidase, although this enzyme is present in rat tissues. Hence, no oxidative degradation of the catecholamines by catechol oxidase occurs *in vivo* as observed in tissue homogenates (Axelrod, 1964).

Pichler & others (1968) found that treatment with 25 mg/kg of the MAO-blocker, pargyline, did not change the formation of THO from ^{3}H-NA, whereas we have found that MAO-blockade by iproniazid markedly decreased the amounts of the substances arising simultaneously with THO formation, viz. VA, DHBA and the unknown fraction, and, concomitantly, in preliminary experiments, also decreased the formation of THO. Although no conclusive explanation can be offered for the discrepancy, it seems possible that pargyline, in the dosage used, did not produce the same degree of MAO inhibition as iproniazid.

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