PRO EXPERIMENTIS

The Purification of Tritiated Noradrenaline

Tritium is used to label a wide variety of compounds of biological interest. The high specific activity obtainable has obvious advantages for facilitating the detection of substances or their metabolic products by means of the measurement of radioactivity, but their decomposition resulting from self-irradiation is a major disadvantage, since it soon impairs the radiochemical purity[1].

We have been interested for some time in the uptake of DL-noradrenaline-7-T by brain slices and in its distribution and metabolism in the central nervous system after i.v. injection. The uptake of radioactivity by brain slices from samples known to be radiochemically pure, when compared with those containing decomposition products, showed that the latter are also readily absorbed by the tissue. Clearly, results of uptake experiments dependant on the measurement of radioactivity would be vitiated by the presence of significant amounts of impurities.

Samples of tritiated noradrenaline with specific activities of up to 10 C/mM were obtained in aqueous solution as the hydrochloride (The Radiochemical Centre, Amersham, England) or as the acetate (New England Nuclear Corporation, Boston, USA). The radiochemical purity of these samples was checked by means of gradient elution chromatography on Whatman P11 cellulose phosphate[2,3] after dilution of 50 μC aliquots with 100 μg of unlabelled noradrenaline as a carrier. The position of the peak representing noradrenaline was determined polarographically[4]. In some of the samples only 50% of the radioactivity was found to be authentic noradrenaline, but whenever low recoveries were encountered examination of the remaining fractions revealed 2 additional radioactive peaks occurring at, or soon after, the hold-up volume of the column, a feature which characterized them as neutral or acidic substances presumably resulting from radiation-induced alteration of the ethanolamine side-chain.

Attempts to separate noradrenaline from these decomposition products by adsorption onto alumina followed by elution with dilute acetic acid eliminated part, but not all, of the impurities. This finding seems to indicate that the catechol moiety remains largely intact, since alumina possesses considerable specificity for catechol derivatives under the conditions employed[5].

Although the foregoing chromatographic procedure is satisfactory for analytical purposes it has the disadvantage that the eluate contains ammonium acetate. For preparative purposes a modification has therefore been devised which avoids this complication and also the necessity for the production of a gradient; in addition the noradrenaline is eluted in a dilute HCl solution; in which form it is both free from ammonium ions and reasonably stable. The addition of sulphite as suggested by AXELROD et al.[6] is unnecessary and best avoided as it interferes with subsequent assay.

Method. Glass columns (0.5 × 35 cm) are packed with a dilute (1 in 20) suspension of Whatman P11 cellulose phosphate (prepared as previously described[2]) in 0.05M ammonium acetate buffer solution (pH 6.0). The suspension is applied by an extension piece 45 cm long. The cellulose powder is allowed to settle by gravity and further packed by pumping buffer through the column at the rate of 0.7 ml/min until no further reduction in the volume of the adsorbent occurs. The sample (up to 2 ml) is added to the column and allowed to enter by gravity. Fractionation is effected by pumping 0.1N HCl through the colum at the rate of about 0.25 ml/min. Fifty 2 ml fractions are collected to obtain complete elution of the purified noradrenaline. An example of the purification of a sample containing 2 mC of DL-noradrenaline-7-T is shown in the Figure; in this experiment the peak containing noradrenaline appeared in fractions 41–45 and accounted for a recovery of 58.5% of the total radioactivity applied to the column. Fluorimetric assay and chromatography by the gradient elution procedure showed that it was at least 98% pure. The impurities appeared as 2 peaks well separated from the noradrenaline: no attempt was made to ascertain whether these 2 radioactive peaks contained more than 1

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3 R. J. MERRILLS and J. OFFERMAN, Biochem. J. *99*, 538 (1966).

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5 F. H. SHAW, Biochem. J. *32*, 19 (1938).

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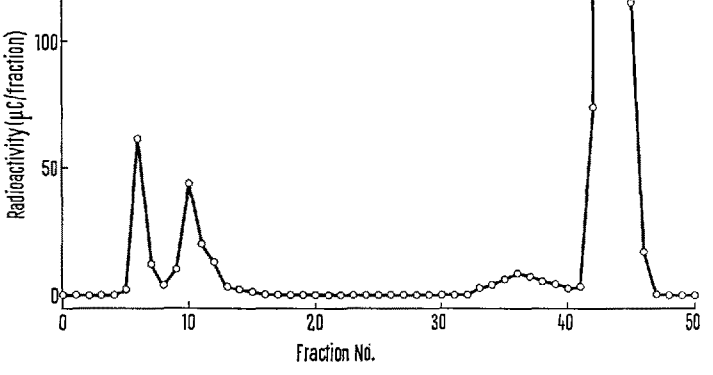

Preparative chromatogram of tritiated noradrenaline on cellulose phosphate. 2 ml of an aqueous solution containing 2 mC (S.A. 10.28 C/mM) was applied to the column. The peak containing the purified noradrenaline appeared in fractions 41–45.

breakdown product, nor have any studies yet been made to assign structural characteristics to them.

The procedure has ensured a supply of radiochemically pure tritiated noradrenaline for tissue uptake and similar investigations. When the solutions are kept at 5 °C subsequent purification has generally been found unnecessary unless they are stored for longer than 3 months, but as a precautionary measure the solutions were analysed by a specific fluorimetric method before use[7].

Zusammenfassung. Eine einfache chromatographische Methode zur Abtrennung von reinem Noradrenalin, welches nicht nur stabil, sondern auch für kritische Stoffwechseluntersuchungen geeignet ist, wird beschrieben.

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Therapeutics Research Division, Pfizer Ltd., Sandwich (Kent, England), 6 June 1968.

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Fluorescence Assay of Tyrosine Hydroxylase Activity in Tissue Homogenate

The enzyme activity of tyrosine hydroxylase found in tissues is so low that radioassay is required. In a radioassay using L-tyrosine-C^{14}, DOPA-C^{14} formed by the enzyme reaction was isolated by an alumina column and measured[1]. The other simpler radioassay is to use L-tyrosine-3, 5-H^3 as a substrate[2]. The unused substrate plus the DOPA formed after the enzyme reaction were trapped on a dowex-50-H^+ column. The tritiated water, which is also a product of the reaction, was collected in the effluent and the radioactivity measured.

It was shown that, when the activity of purified tyrosine hydroxylase was assayed by the appearance of DOPA from L-tyrosine with the fluorometric procedure, the values were essentially the same as calculated from radioassay[1]. In the fluorometry, DOPA was isolated by an alumina column, and measured spectrofluorometrically after the trihydroxyindole procedure. We tried to apply this fluorescence assay to the measurement of tyrosine hydroxylase activity in crude tissue preparations such as homogenate. When the homogenate of adrenal glands or brain was used as an enzyme preparation, a large blank value in the enzyme assay was observed. In the case of adrenal glands, a large amount of catecholamines in the homogenate was also adsorbed on the alumina column, and interferred with the spectrofluorometric assay of DOPA after the trihydroxyindole procedure. Therefore, in order to measure tyrosine hydroxylase activity in crude tissue preparations fluorometrically, it is necessary to remove interferring substances. A method of fluorometric assay of tyrosine hydroxylase activity in the homogenate of adrenal glands and brain is reported in this communication.

Adrenal glands and brain (caudate nucleus and brain stem) were freshly removed from rabbits. Tissue homogenate was prepared in 0.25 *M* sucrose with glass, motor-driven homogenizers. Purified tyrosine hydroxylase was prepared from bovine adrenal medulla[1]. Incubation mixture contained: 200 µmoles acetate buffer (pH 6.0), 400 mµmoles L-tyrosine, 100 µmoles mercaptoethanol, 1 µmole 2-amino-4-hydroxy-6,7-dimethyl-5, 6, 7, 8-tetrahydro-pteridine[3], an appropriate amount of the enzyme preparation (homogenate or purified enzyme), and water to 1.0 ml. The incubation was carried out at 30 °C[4] for 15 min in a metabolic shaker. For the blank incubation, D-tyrosine was used as substrate instead of L-tyrosine. 10 mµmoles of DOPA were added to another blank incubation as internal standard. Reaction was stopped by the addition of 50 µl of glacial acetic acid. The incubation mixture was centrifuged to remove protein. The precipitate was washed with 1 ml of 2% acetic acid and centrifuged. The combined supernatant was passed through 2 columns fitted together in piggyback fashion; the top column containing Florisil (100/200 mesh, 0.6 × 4.0 cm), which had previously been washed with 2% acetic acid until the effluent was pH 4.0[5], and the bottom column containing Amberlite CG-120-Na^+ (Type I, 0.6 × 4.0 cm), which had previously been washed with 5 *N* NaOH and water. The effluent through the 2 columns were discarded. Both columns were washed with 5 ml of 2% acetic acid successively, and the washings were discarded. DOPA was passed through the first Florisil column and adsorbed on the second Amberlite CG-120 column. The second Amberlite-CG-120 column was separated and washed with 5 ml of water. DOPA was eluted with 10 ml of 0.1 *M* sodium acetate buffer (pH 6.5) into a beaker containing 0.5 ml of 0.2 *M* EDTA. Catecholamines were retained on the column. 400 mg of alumina, which had been previously treated with acid, was added into the beaker[6]. The pH was adjusted to 8.5 with the glass electrode by the dropwise addition of 3 *N* NH_4OH with constant stirring, the alumina was allowed to settle and the aqueous phase was decanted and discarded. The alumina was transferred to a column (0.6 cm in diameter) and washed with 10 ml of water. After washing, the DOPA was eluted with 2 ml of 0.3 *N* acetic acid, and assayed spectrofluorometrically by trihydroxyindole procedure[7,8]. To 1.0 ml of the eluate were added 1.0 ml of 1 *M* sodium acetate and 0.1 ml of 0.25% $K_3Fe(CN)_6$. After 3 min, 1.0 ml of a mixture of 2% ascorbic acid/20% NaOH (1/9, v/v) was added. As an external standard and a reagent blank, 5 mµmoles of DOPA in 1.0 ml of 0.3 *N* acetic acid and 1.0 ml of 0.3 *N* acetic acid were treated at the same time. The fluorescence

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