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.

J. L. Offerman and R. J. Merrills

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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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[3] Pteridine cofactor was generously supplied by Dr. D. E. Wolf (Merck Institute for Therapeutic Research, Rahway), which is gratefully acknowledged.

[4] The optimum temperature for tyrosine hydroxylase activity in this incubation mixture was found to be at 30 °C. S. Ayukawa, T. Takeuchi, T. Sezaki, H. Hara, H. Umezawa and T. Nagatsu, J. Antibiotics *21*, 350 (1968).

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was measured at 480 nm with the excitation light at 356 nm using an Aminco-Bowman spectrophotofluorometer[9]. The DOPA formed enzymatically was calculated from the value of internal standard by the following equation. $[F(L) - F(D)]/[F(D + IS) - F(D)] \times 10$ mμmoles, where F(L) = reading of L-tyrosine incubation, F(D) = reading of D-tyrosine incubation, and F(D + IS) = reading of D-tyrosine plus DOPA (internal standard, 10 mμmoles) incubation.

In this procedure, DOPA is isolated specifically. The blank value was less than 1 mμmole of DOPA, when homogenate containing 33 mg (wet weight) of adrenal glands or caudate nucleus was used. Overall recovery of internal standard DOPA was 40 ± 1 (S.D.) % (n = 10), and constant. Limit of the sensitivity was about 1 mμmole DOPA formed enzymatically.

It was found in later experiments that the first Florisil column could be omitted. In this case, the reaction was stopped with 50 μl of 50% trichloroacetic acid. The incubation mixture was centrifuged. The precipitate was washed with 1 ml of water and recentrifuged. The combined supernatant was passed through an Amberlite CG-120-Na^+ (Type I, 0.6 × 4.0 cm) column. The column was washed with 5 ml of water. Subsequent procedures were the same as described above. This method gave higher blank value, but the recovery of DOPA was 60%, which was reproducible.

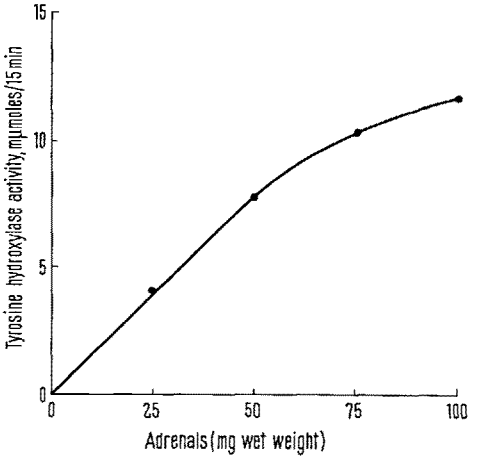
As shown in the Figure, the reaction rate measured by using sucrose homogenate was linear up to 50 mg of rabbit adrenals. Tyrosine hydroxylase activity in adrenal glands and brain were shown in the Table. This fluorescence assay could easily be applied to the measurement of the activity of purified adrenal tyrosine hydroxylase. In one experiment, 12.2 mμmoles DOPA were found by the fluorometry, and 12.3 mμmoles by the radioassay[1] in which DOPA-C^{14} was measured from L-tyrosine-C^{14}. This showed that the appearance of DOPA with this fluorometric procedure is essentially the same as calculated from the radioassay.

Although fluorescence assay is less sensitive than radioassay, the enzyme activity in homogenate of such tissues as adrenal glands or brain can be measured exactly. Fluorescence assay has some advantages. Besides the convenience that a labelled substrate and a liquid scintillation spectrometer are dispensable, separate measurement of tyrosine concentration in the homogenate is not necessary for the calculation[10].

Tyrosine hydroxylase activity in homogenates of rabbit organs measured by the fluorescence assay

Organs	Activity (mean ± S.D.) mμmoles/g tissue per h
Adrenal gland (6)	431 ± 21
Brain	
Caudate nucleus (5)	284 ± 97
Brain stem (1)	68

No. of organs in brackets.



Tyrosine hydroxylase activity as a function of enzyme amount. Sucrose homogenate of rabbit adrenal glands was used as enzyme. Incubation was for 15 min at 30 °C. The DOPA formed was isolated and assayed spectrofluorometrically as described in the text.

Zusammenfassung. Es wird eine Fluoreszenzmethode zur Bestimmung der Tyrosin-Hydroxylase-Aktivität von Homogenat beschrieben, die auf der Spektrofluorometrie der DOPA-Bildung beruht. Die Tyrosin-Hydroxylase-Aktivität von Homogenat der Nebenniere und des Gehirns (Nucleus caudatus) wurde mit dieser Methode gemessen.

T. NAGATSU and T. YAMAMOTO

Department of Biochemistry, School of Dentistry, Aichi-Gakuin University, Nagoya (Japan), 3 July 1968.

[9] The Aminco-Bowman Spectrophotofluorometer was purchased by United States Public Health Service Research Grant No. 7 R05 TW-00219-01A1.

[10] The authors would like to thank Prof. H. UMEZAWA, Dr. T. TAKEUCHI, Dr. K. HARA and Mr. T. AYUKAWA (Institute of Microbial Chemistry, Tokyo) for their kind help in conducting some part of the experiment.

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