REFERENCES

- Ames, S. R. & Risley, H. A. (1948). *Proc. Soc. exp. Biol., N.Y.*, **69**, 267.
- Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1950). *Biochem. J.* **46**, 426.
- Bate-Smith, E. C. (1949). *Biochem. Soc. Symp.* no. 3, 62.
- Bate-Smith, E. C. & Westall, R. G. (1950). *Biochim. Biophys. Acta*, **4**, 427.
- Calvery, H. O. (1944). *The Chemistry of the Amino Acids and Proteins*, ed. C. L. A. Schmidt, 2nd ed., p. 185. Springfield and Baltimore: Thomas.
- Chargaff, E., Levine, C. & Green, C. (1948). *J. biol. Chem.* **175**, 67.
- Dent, C. E. (1948). *Biochem. J.* **43**, 169.
- Ekman, B. (1948). *Acta chem. scand.* **2**, 383.
- James, W. O. (1948). *Nature, Lond.*, **161**, 851.
- Kelemen, E., Tanos, B. & Halmagyi, D. (1950). *Biochem. J.* **47**, 138.
- Martin, A. J. P. (1949). *Biochem. Soc. Symp.* no. 3, 4.
- Maw, G. A. (1948). *Biochem. J.* **43**, 139.
- Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
- Steward, F. C. & Thompson, J. F. (1950). *Ann. Rev. Plant Physiol.* **1**, 233.
- Tabone, J. & Robert, D. (1948). *Ann. Inst. Pasteur*, **74**, 146.
- Tabone, J., Robert, D. & Troestler, J. (1948). *Bull. Soc. chim. biol., Paris*, **30**, 547.
- Urbach, K. F. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 146.

β -(3:4-Dihydroxyphenyl)ethylamine (hydroxytyramine) in Normal Human Urine

BY U. S. VON EULER, U. HAMBERG AND S. HELLNER
Physiology Department, Faculty of Medicine, Karolinska Institute, Stockholm

(Received 16 March 1951)

Following the discovery by Holtz, Heise & Lüdtke (1938) of L-dopadecarboxylase in animal tissues, Holtz, Credner & Koepp (1942) demonstrated that after administration of 3:4-dihydroxyphenylalanine (DOPA) to man, guinea pigs, rabbits and cats, the urine contained considerable quantities of hydroxytyramine present in free and conjugated form. They also obtained some evidence for the presence normally of hydroxytyramine in urine. Holtz, Credner & Kroneberg (1947) later showed that normal urine contained what appeared to be a mixture of hydroxytyramine, adrenaline and noradrenaline (urosympathin). In a recent paper, however, Kroneberg & Schümann (1950) express the opinion that in man and certain animals noradrenaline forms most if not all of the catechol amines in urine.

The presence in normal urine of adrenaline and noradrenaline could be demonstrated biologically after adsorption on aluminium hydroxide (von Euler & Hellner, 1951 *a, b*). When purified urine extracts were submitted to paper chromatography, a mixture of *n*-butanol and *N*-hydrochloric acid being used as a solvent, not only noradrenaline and adrenaline were found, but also considerable quantities of another catechol amine occupying the site of hydroxytyramine on the chromatogram. The present paper deals with the separation and identification of hydroxytyramine from extracts of human urine by use of partition chromatography on a starch column.

EXPERIMENTAL

Preparation of extracts. Urine was obtained from medical students. The catechol amines were separated by adsorption in alumina in portions of 0.5–2 l. according to the technique described previously (von Euler & Hellner, 1951 *a*). In order to facilitate filtration of the alumina precipitate Hyflo Supercel (Johns Manville and Co.) was added when necessary in amounts of 30–50 g./l.

In two preparations smaller portions of whole urine were boiled for 20 min. at pH 2 in order to split conjugated catechol amines (von Euler & Hellner, 1951 *a*). This procedure generally increased the yield of biologically active catechol amines by 30–100%, presumably by splitting ethereal sulphates (Richter, 1940). The glucuronides (Clark, Akawie, Pogrund & Geissman, 1950) would presumably not be split by this mild hydrolysis, which, however, was chosen in order to minimize the inactivation of the free catechol amines (by racemization). No qualitative differences were observed between untreated and hydrolysed urine.

Partition chromatography on starch column. The catechol amines of the urine extract were taken up in *n*-butanol by repeated evaporation of the aqueous solution with *n*-butanol at low temperature *in vacuo*. The butanol extract thus obtained was suitably concentrated and used for partition chromatography on a starch column with *n*-butanol/*N*-HCl as solvent according to the technique used by Hamberg & von Euler (1950). Each 2 hr. sample was evaporated to dryness *in vacuo* and dissolved in 2 ml. of water. These solutions were used for colorimetric determination, paper chromatography and biological assay. A control run was made with a mixture of adrenaline, noradrenaline and hydroxytyramine, together with urine and the *R* values determined.

Colorimetric determination. Adrenaline and noradrenaline were estimated quantitatively by the colorimetric method of von Euler & Hamberg (1949). Hydroxytyramine was determined by essentially the same method, the iodine treatment being extended to 3 min. at pH 6.7.

Paper chromatography. Filter paper runs were made on Grycksbo OB paper with *n*-butanol/*N*-HCl as solvent for 20–30 hr. at 25°. The spots were developed with a weak solution of ferricyanide. The location of the spots was compared with those obtained with pure standard solutions.

Biological assay. The samples were assayed on the blood pressure of the cat and compared with values obtained using standard solutions. The different type of blood pressure effects produced by the three substances in question facilitated the identification. The different substances were localized by the *R* values and estimated colorimetrically. For comparison the hydrochlorides of the synthetic hydroxytyramine, (–)-noradrenaline and (–)-adrenaline were used.

Fig. 1. Paper chromatogram, normal human urine before hydrolysis. Solvent, *n*-butanol/*N*-HCl. 25 hr. at 25°. (1) Normal urine + noradrenaline and adrenaline. (2) Normal urine. (3) Noradrenaline, adrenaline and hydroxytyramine.

RESULTS

In a preliminary run the localization of noradrenaline, adrenaline and hydroxytyramine was ascertained by adding the pure substances to an extract of

hydrolysed urine which had been transferred into butanol. The amounts added were rather higher than those estimated to occur in the amount of urine used for this experiment (about 2 l.), thereby allowing a definite localization.

The following *R* values were obtained: hydroxytyramine, 0.31; adrenaline, 0.25; noradrenaline, 0.16.

Results obtained with hydrolysed urine

Pooled urine was treated with acid as described above, and a butanol extract prepared. The primary extracts were readsorbed on alumina in order to obtain further purification, although this entailed some losses. Butanol solution of the urine extract (2 ml.) corresponding to 4.6 l. of urine was applied to the column. Colorimetric tests revealed two different groups of active samples with *R* values corresponding to hydroxytyramine and noradrenaline. The latter samples were subjected to paper chromatography and showed spots in the position typical of noradrenaline.

Fig. 2. Comparison of the effects on the blood pressure of the chloralosed cat of (1) authentic hydroxytyramine with (2) and (3). Material obtained from human urine. (1) 40 µg. hydroxytyramine (HOT). (2) Colorimetric equivalent of 5 µg. HOT (sample 26). (3) Colorimetric equivalent of 35 µg. HOT (sample 26).

As also observed on paper chromatograms with the urine extract, hydroxytyramine forms the largest fraction of the catechol amines (Fig. 1). The blood pressure response given by the 26 hr. sample showed a good agreement quantitatively and qualitatively with that given by authentic hydroxytyramine (Fig. 2). The total yield colorimetrically was 148 µg. estimated as hydroxytyramine.

Table 1. *R* values and results of biological and colorimetric estimation of samples of catechol amines obtained from a starch column; first experiment

Sample no.	Paper chromatography	Estimated		<i>R</i> value
		Colorimetrically Equiv. to 100 µg. hydroxytyramine	Biologically Equiv. to 120 µg. hydroxytyramine	
26	—	+ (<20 µg.)	Noradrenaline	0.35
52–58	Noradrenaline			0.18

Table 2. *R* values and results of biological and colorimetric estimation of fractions of urinary catechol amines obtained from a starch column; second experiment

Sample no.	Paper chromatography	(Hydrolysed material.)			<i>R</i> value
		Estimated			
		Colorimetrically	Biologically		
26-28	Hydroxytyramine	108 μ g. hydroxytyramine	Hydroxytyramine		0.36
34	—	—	Adrenaline		0.27
44-48	—	—	Noradrenaline		0.19

When repeated on a second preparation of urine obtained in the same way the results were the same as shown by the *R* values. In addition, evidence was obtained for the occurrence of adrenaline in this preparation.

Unhydrolysed urine

In order to ascertain whether urine contains hydroxytyramine in addition to adrenaline and noradrenaline in a preformed state, an experiment similar to those described above was undertaken with the difference that the urine was not boiled at pH 2 prior to the adsorption on alumina.

The results were essentially the same. Hydroxytyramine was found in a group of samples (*R* value, 0.36) and estimated colorimetrically to be 144 μ g. In paper chromatograms adrenaline and noradrenaline were demonstrated in samples giving *R* values 0.28 and 0.19 respectively, i.e. in good agreement with the results obtained on hydrolysed urine.

DISCUSSION

The original claim of Holtz *et al.* (1942) that hydroxytyramine is normally excreted in urine has been confirmed. The doubts as to the normal occurrence of hydroxytyramine in normal human urine, which Kroneberg & Schümann (1950) recently expressed, have therefore not been substantiated. On the contrary, hydroxytyramine is, quantitatively, the most important catechol amine present. The earlier results of Holtz *et al.* (1942) that administration of DOPA leads to a considerable excretion of hydroxytyramine in urine gives indication that at least part of the hydroxytyramine found normally in the urine may be derived from DOPA by decarboxylation in the body.

As shown by Goodall hydroxytyramine occurs in sheep heart (1950*a*) and in suprarenal extracts (1950*b*), which is of interest since suprarenals may contain L-dopadecarboxylase in considerable amounts as shown by Langemann (1950). Goodall (1950*b*) also has obtained evidence by paper chromatography for the presence of DOPA in suprarenal extracts of thyroidectomized sheep. Bing (1940) has shown that hydroxytyramine is formed during perfusion of the isolated kidney with DOPA. In perfusates of isolated kidneys Williams &

Grossman (1938) demonstrated the presence of a substance with the properties of hydroxytyramine. It is therefore not unlikely that the kidney, being rich in dopadecarboxylase, participates in the formation of hydroxytyramine.

The relatively large quantities of hydroxytyramine may interfere with the biological assay of other catechol amines in urine. The relative amount of hydroxytyramine may be estimated to be about five times that of noradrenaline. The pressor effect on the blood pressure of the cat is, therefore, still largely determined by noradrenaline, since this substance is fifty to hundred times more active than hydroxytyramine on the blood pressure of the cat. On the hen rectal caecum also the activity of hydroxytyramine is much less (10–20 times) than that of noradrenaline. Even if the hydroxytyramine content of the urine should amount to ten times that of noradrenaline, the apparent 'adrenaline' figure due to hydroxytyramine would not exceed 10% of the total catechols under the rather unfavourable assumption that the activity ratios of adrenaline and noradrenaline are as low as ten on the hen rectal caecum. As a rule the apparent 'adrenaline' figure caused by the hydroxytyramine would be less than 5% of the total catechol amines, and since the figures generally found in urine are of the order of 15–30%, the error will not be serious. On the other hand, if the noradrenaline figure is low and disturbances occur in the catechol amine distribution, the error may become appreciable.

SUMMARY

1. By partition chromatography on starch columns of human urine extracts hydroxytyramine, adrenaline and noradrenaline have been separated and identified.

2. The fractions with *R* value corresponding with those of hydroxytyramine give a characteristic spot in paper chromatograms and show the same behaviour as a hydroxytyramine on colorimetric estimation and biological assay.

3. Hydroxytyramine occurs largely in the free state in urine.

4. The daily output of hydroxytyramine is estimated at 0.1–0.2 mg. and exceeds considerably that of noradrenaline and adrenaline.

REFERENCES

- Bing, R. J. (1940). *Amer. J. Physiol.* **132**, 497.
 Clark, W. G., Akawie, R. I., Poggrund, R. S. & Geissman, T. A. (1950). *XVIIIth Int. physiol. Congr. Abstr.* p. 159.
 Euler, U. S. von & Hamberg, U. (1949). *Science*, **110**, 561.
 Euler, U. S. von & Hellner, S. (1951*a*). *Acta physiol. scand.* **22**, 161.
 Euler, U. S. von & Hellner, S. (1951*b*). (To be published.)
 Goodall, McCh. (1950*a*). *Nature, Lond.*, **166**, 738.
 Goodall, McCh. (1950*b*). *Acta chem. scand.* **4**, 550.
 Hamberg, U. & von Euler, U. S. (1950). *Acta chem. scand.* **4**, 1185.
 Holtz, P., Credner, K. & Koepp, W. (1942). *Arch. exp. Path. Pharmacol.* **200**, 356.
 Holtz, P., Credner, K. & Kroneberg, G. (1947). *Arch. exp. Path. Pharmacol.* **204**, 228.
 Holtz, P., Heise, R. & Lüdtkke, K. (1938). *Arch. exp. Path. Pharmacol.* **191**, 87.
 Kroneberg, G. & Schümann, H. J. (1950). *Arch. exp. Path. Pharmacol.* **209**, 350.
 Langemann, H. (1950). *XVIIIth Int. physiol. Congr. Abstr.* p. 325.
 Richter, D. (1940). *J. Physiol.* **98**, 361.
 Williams, J. R. & Grossman, E. B. (1938). *Amer. J. Physiol.* **123**, 364.

The Cytochrome System of Adrenal Medulla

By C. L. TSOU

Molteno Institute, University of Cambridge

(Received 19 March 1951)

Huszak (1932, 1942) examined the cytochrome system of the medulla of the adrenal gland and reached the conclusion that, unlike all other animal tissues, it does not contain a cytochrome oxidase. The oxidation of its reduced cytochrome was said to be carried out by a peroxidase, together with hydrogen peroxide produced by the autoxidation of substances such as ascorbic acid or adrenaline. In the present investigation, however, it is shown that the adrenal medulla contains a perfectly normal cytochrome oxidase.

EXPERIMENTAL

Adrenal medulla preparation. Adrenal glands from ten or twelve horses at a time were obtained directly from the slaughter-house. The medulla (10 g. wet weight) was carefully cleaned from all traces of cortex, cut into small pieces about $5 \times 5 \times 1$ mm., and rapidly homogenized for 3 min. in a Waring blender with 100 ml. of cold 0.02 M-phosphate buffer, pH 7.3. The homogenate was then centrifuged at 2000 rev./min. for 10 min. and the supernatant was again centrifuged at 12,000 rev./min. for 25 min. The precipitate from the latter operation was then washed twice with 50 ml. of cold 0.01 M-phosphate buffer, pH 7.3, collected by centrifugation (12,000 rev./min., 25 min.) after each washing, and the final precipitate was suspended in an equal volume of 0.1 M-phosphate buffer, pH 7.3.

Cytochrome *c* was prepared by the method of Keilin & Hartree (1945) and contained 0.34% Fe.

Ferrocycytochrome *c* was prepared immediately before use by reducing ferricytochrome *c* in 0.1 M-phosphate buffer, pH 7.3, with a current of hydrogen in presence of platinum black. Ferrocycytochrome *c* thus prepared was slowly oxidized by the H_2O_2 formed in the solution from the Pt-catalysed reduction

of dissolved O_2 by H_2 . To prevent this oxidation, a few drops of dilute catalase solution were added immediately after the current of H_2 had been stopped. The Pt black was then removed by filtration.

Catalase was prepared by the method of Keilin & Hartree (1937).

Oxidation of ferrocycytochrome *c*. The oxidation of ferrocycytochrome *c* by the washed medulla preparation was followed by measuring the intensity of its α -band at 550 m μ . in a Beckman spectrophotometer. Each 1 cm. cell contained 1.7×10^{-5} M-ferrocycytochrome *c*; 0.1 M-phosphate buffer, pH 7.3; 0.1–0.2 mg. enzyme preparation; and 1 μ g. catalase in a total volume of 3 ml. With gas mixtures other than air, 2 cm. cylindrical cells, provided with ground-glass stoppers, were used. These cells, with a total capacity of 5.5 ml., contained the same concentration of all the above reagents except that the cytochrome concentration was 0.85×10^{-5} M. The required gas mixture was bubbled through the solution in these cells in presence of a drop of capryl alcohol before the addition of the enzyme. The concentration of ferrocycytochrome at time *t* is given by $(D_t - D_\infty)/(1.92 \times 10^4)$, where D_t and D_∞ are the density at time *t* and after complete oxidation respectively and 1.92×10^4 is the difference between the molecular extinction coefficients of reduced and oxidized cytochrome *c* at 550 m μ .

The activities of cytochrome oxidase, succinic dehydrogenase, succinic oxidase and dihydrocozymase oxidase were measured as described by Slater (1949*a*, *b*, 1950).

Peroxidase activity was measured either as described by Keilin & Mann (1937) or by a spectrophotometric method of Morell (1951).

RESULTS

Spectroscopic observations. Intact adrenal medulla tissue, as well as the washed preparation, showed a diffuse *d*-band of cytochrome at 530 m μ . and a