An Enzymic Method for the Estimation of L-Tryptophan

By T. A. SCOTT

Department of Biochemistry, The University of Leeds

(Received 15 February 1961)

Strictly specific, but time-consuming, microbiological assay procedures for L-tryptophan have been published (Dunn, Schott, Frankl & Rockland, 1945; Schweigert, Sauberlich & Elvehjem, 1946). The chemical method of Udenfriend & Peterson (1957), with nitrite and *p*-dimethylaminobenzaldehyde, lacks specificity. Egelhaaf (1957) has reported a very sensitive chromatographic method, in which the tryptochrome complex is measured fluorimetrically. The procedure is rather lengthy and has an error of $\pm 10\%$.

The enzymic determination described here is strictly specific for L-tryptophan, suitable for quantities as low as $1\mu\text{g.}$ and fairly rapid.

L-Tryptophan is converted almost quantitatively into indole, pyruvic acid and ammonia by the enzyme tryptophanase under optimum conditions. The indole is determined colorimetrically with *p*-dimethylaminobenzaldehyde or *p*-dimethylaminocinnamic aldehyde, after extraction from incubation mixtures with light petroleum (b.p. $40\text{--}60^\circ$). This indole determination is a modification of the original method of Happold & Hoyle (1934) for the measurement of indole in bacterial cultures.

MATERIALS AND APPARATUS

Extraction funnels. These funnels (250 ml. capacity) should be of a narrowly tapering pear shape, so that a 40 ml. graduation mark can be made accurately.

Tryptophanase preparation. An acetone-dried powder of *Escherichia coli* cells possessing tryptophanase activity was prepared according to Dawes, Dawson & Happold (1947). The acetone-dried cells keep indefinitely at -15° . The acetone-dried powder (0.2 g.) was extracted at room temperature for 1 hr. with 10 ml. of water and centrifuged, and the clear, straw-coloured supernatant retained.

Pyridoxal phosphate. This was an aqueous 0.02% solution, stored in the dark at 4° .

Buffer. KH_2PO_4 (18 g./l.) was adjusted to pH 7.8 with 5N-NaOH.

Trichloroacetic acid. This was an aqueous 50% (w/v) solution.

Dimedone. This was an ethanolic 1% (w/v) solution.

***p*-Dimethylaminobenzaldehyde reagent.** A.R. *p*-dimethylaminobenzaldehyde (16 g.) was dissolved in 1.25 l. of absolute ethanol and 320 ml. of conc. HCl.

***p*-Dimethylaminocinnamic aldehyde reagent.** *p*-Dimethylaminocinnamic aldehyde (2.34 g.) was dissolved in 780 ml. of ethanol and 100 ml. of conc. HCl and water to 1 l. (Turner, 1959).

EXPERIMENTAL AND RESULTS

Partition of indole between water and light petroleum

Standard, aqueous indole solutions (25 ml.) were shaken at 20° with 25 ml. of light petroleum (b.p. $40\text{--}60^\circ$). Equilibration was complete in 30 sec. Indole was determined in the aqueous phase by the absorption at $275\text{ m}\mu$ (Unicam SP. 500 spectrophotometer, 1 cm. light-path), with water equilibrated with an equal volume of light petroleum at the blank. The calibration curve was linear up to an extinction of 0.84, which corresponded to 1.84 mg. of indole/100 ml.

The results shown in Table 1 give an average value of 5.01 for the partition coefficient in favour of the light petroleum. About 17% of the indole is left in the aqueous phase and so standardization of the volumes of aqueous and organic phases is necessary during the extraction procedure.

Reaction of indole with pyridoxal phosphate

All incubation mixtures contain pyridoxal phosphate as the coenzyme of tryptophanase. Under the acid conditions produced when trichloroacetic acid is used to precipitate the protein, indole and pyridoxal phosphate (or pyridoxal) react to form a red pigment. This loss of indole can be prevented by the addition of dimedone, which combines with the pyridoxal phosphate and renders it unavailable for reaction with the indole.

Table 1. *Partition coefficient of indole between light petroleum and water*

Aqueous indole was equilibrated at 20° with an equal volume of light petroleum (b.p. $40\text{--}60^\circ$); indole in the aqueous phase was determined by absorption at $275\text{ m}\mu$.

Initial concn. of indole in aq. phase (mg./100 ml.)	Concn. of indole in aq. phase after equilibration (mg./100 ml.)	Concn. of indole in light petroleum after equilibration (calc. by difference) (mg./100 ml.)	Partition coeff.
0.53	0.09	0.44	4.88
0.98	0.16	0.82	5.13
1.65	0.27	1.38	5.11
1.65	0.28	1.37	4.93
Average partition coefficient			5.01

The exact nature of the red pigment formed from indole and pyridoxal phosphate is unknown, but phosphate is absent.

Colorimetric reagents

Formation of the 'roseindole' colour between indole and *p*-dimethylaminobenzaldehyde (Fischer, 1886) is complete within a few seconds. After reaching a peak value, the extinction (measured at $\lambda_{\max}$, 570 $m\mu$, see Fig. 1) decreases at the rate of 2.9 %/min. (see Fig. 2). In practice, the colour

formed was read exactly 5 min. after adding this reagent.

p-Dimethylaminocinnamic aldehyde produces a green colour ($\lambda_{\max}$, 625 $m\mu$). Colour development is complete in 15 min. and the maximum intensity remains constant for several hours (see Fig. 2).

With the same amount of indole, the intensity of absorption (at 625 $m\mu$) with the latter reagent is approximately 2.5 times that with the former (at 570 $m\mu$).

Procedure

Equal volumes of pyridoxal phosphate solution and tryptophanase preparation are incubated for 10 min. at 37°. This mixture (2 ml.) is added to 3 ml. of test tryptophan solution and 5 ml. of phosphate buffer.

Incubation is continued for 30 min. at 37°. Dimedone reagent (1 ml.), followed by 5 ml. of trichloroacetic acid solution, is then added. After 3–4 min., the coagulated protein is filtered off through a plug of absorbent cotton wool in a 1 in. diameter glass funnel. The filtrate passes directly into 40 ml. of light petroleum contained in an extraction funnel, and the incubation tube and filter are washed with water until the aqueous layer in the funnel reaches the 40 ml. graduation mark. Organic and aqueous phase are shaken vigorously together for 30 sec. and allowed to separate. The bottom, aqueous layer is run off and, to avoid error, any residual water on the sides of the funnel is removed by repeating the shaking process.

p-Dimethylaminobenzaldehyde reagent (10 ml.) or *p*-dimethylaminocinnamic aldehyde reagent (10 ml.) is added to the light-petroleum layer and shaken vigorously for a few seconds. The lower, coloured reagent layer is removed and, if necessary, clarified by low-speed centrifuging or rapid filtration through absorbent cotton wool.

Colours are read at $\lambda_{\max}$ against a reagent blank (Unicam SP. 600 spectrophotometer, 1 cm. light-path).

With *p*-dimethylaminobenzaldehyde reagent, the standard curve is linear up to E 0.6 at 570 $m\mu$, which corresponds to 60 μg . of L-tryptophan. With *p*-dimethylaminocinnamic aldehyde reagent, the standard curve is linear up to E 0.76 at 625 $m\mu$, which corresponds to 30 μg . of L-tryptophan.

Error

Twenty separate estimations were performed, each on a different day, with the same standard L-tryptophan solution of 60 μg . of L-tryptophan/3 ml., with the *p*-dimethylaminobenzaldehyde reagent. The mean value for L-tryptophan was 59.8 μg ., with an S.D. of $\pm 2.22 \mu\text{g}$.

Fig. 1. Absorption spectra of 'roseindoles'. ---, Indole (32 μg .) extracted from aqueous solution with equal volume of light petroleum (b.p. 40–60°) and colour developed with 10 ml. of *p*-dimethylaminobenzaldehyde reagent, $\lambda_{\max}$, 570 $m\mu$, scanning time 5 min.; —, indole (22 μg .) treated similarly and colour developed with *p*-dimethylaminocinnamic aldehyde reagent, $\lambda_{\max}$, 625 $m\mu$, read after 15 min.

Fig. 2. Development and decay of indole colour complexes at 20°. ●, Development of green colour produced from indole and *p*-dimethylaminocinnamic aldehyde reagent, measured at 625 $m\mu$; ○, decay of red colour produced from indole and *p*-dimethylaminobenzaldehyde reagent, measured at 570 $m\mu$.

DISCUSSION

The method was originally developed for the measurement of L-tryptophan in growing bacterial cultures. It has since been used successfully for determining L-tryptophan in the intracellular, free amino acid pool of *Escherichia coli* and in *Calliphora erythrocephala* during development from larva to imago (Scott, 1960), and in protein hydrolysates (Toothill, 1961), so it appears generally applicable to biological material. Tryptophanase is a sulphydryl enzyme (Happold & Turner, 1960) and, when oxidizing agents are present in the solution under test it would seem expedient to protect the enzyme by the addition of glutathione. This has never been found necessary, however, by the author.

Tryptophanase is strictly specific for L-tryptophan and converts no other known, naturally occurring compound into indole or indole derivatives. If test solutions already contain indole (or other substances soluble in light petroleum and capable of colour production with either of the colorimetric reagents), this can be removed first by light-petroleum extraction.

SUMMARY

1. An enzymic method is described for the estimation of L-tryptophan in biological material, in the range 1–60 μ g.

2. L-Tryptophan is converted quantitatively into indole by the enzyme tryptophanase, and the indole measured colorimetrically, after extraction with light petroleum, with either *p*-dimethylaminobenzaldehyde or *p*-dimethylaminocinnamic aldehyde reagents.

This work was supported by a grant from the D.S.I.R. The author is grateful for the encouragement of Professor F. C. Happold, upon whose own research this present paper is based.

REFERENCES

- Dawes, E. A., Dawson, J. & Happold, F. C. (1947). *Nature, Lond.*, **159**, 99.
 Dunn, M. S., Schott, H. F., Frankl, W. & Rockland, L. B. (1945). *J. biol. Chem.* **157**, 387.
 Egelhaaf, A. (1957). *Z. Naturf.* **12b**, 465.
 Fischer, E. (1886). *Ber. dtsh. chem. Ges.* **19**, 2988.
 Happold, F. C. & Hoyle, L. (1934). *Biochem. J.* **28**, 1171.
 Happold, F. C. & Turner, J. (1960). *Nature, Lond.*, **185**, 763.
 Schweigert, B. S., Sauberlich, H. E. & Elvehjem, C. A. (1946). *J. biol. Chem.* **164**, 213.
 Scott, T. A. (1960). Ph.D. Thesis: University of Leeds.
 Toothill, C. (1961). Ph.D. Thesis: University of Leeds.
 Turner, J. M. (1959). Ph.D. Thesis: University of Leeds.
 Udenfriend, S. & Peterson, R. E. (1957). In *Methods in Enzymology*, vol. 3, p. 890. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Biochem. J. (1961) **80**, 464

Phospholipids in Ram Semen: Metabolism of Plasmalogen and Fatty Acids

By E. F. HARTREE* AND T. MANN

A.R.C. Unit of Reproductive Physiology and Biochemistry, Moltano Institute and Department of Veterinary Clinical Studies, University of Cambridge

(Received 23 December 1960)

Previous studies on the nature and function of lipids in ram semen led us to conclude that the phospholipids of spermatozoa consist mainly of choline-based plasmalogen, and that sperm respiration in the absence of seminal fructose is largely dependent on the oxidation of fatty acids that arise from hydrolysis of this plasmalogen (Lovern, Olley, Hartree & Mann, 1957; Hartree & Mann, 1959, 1960a).

The present paper is an extension of these studies, the results of which fall under three headings: (1) A comparison has been made

* Present address: Animal Research Station, 307 Huntingdon Road, Cambridge.

between the components of the phospholipid fraction extracted from freeze-dried spermatozoa and those of corresponding fractions obtained from fresh, or frozen and thawed, spermatozoa. This comparison was prompted by the appearance of papers by Gray (1960a) and by Wren (1960). Gray produced evidence that the phosphatide fraction extracted from frozen whole ram semen contained lecithin and plasmalogen in approximately equal amounts. Wren drew attention to the widespread occurrence in tissues of lipoamino acids and lipo-peptides and concluded that such lipid derivatives may be artifacts when prepared from freeze-dried material. (2) Direct quantitative evidence for the