NaOH. The water layer was separated and evaporated to dryness in a current of warm air. The resulting solid was recrystallized from ethanol, giving a 20% yield of the ethanol compound (Found: Na, 13.5. Calc. for $C_4H_7O_2Na$, C_4H_5OH : Na, 13.4%). $[\alpha]_D -14.6^\circ$ [expected, -14.4° ; Lehninger & Greville (1953), who also quote earlier authors]. The ethanol could be removed by dissolving the crystals in water, evaporating and finally drying in a vacuum desiccator.

DISCUSSION

The main loss in the preparative procedure appears to occur during the hydrazinolysis. The conversion into sodium β -hydroxybutyrate gave a poor yield simply because no attempt was made to retrieve the salt from the mother liquor after recrystallization from ethanol. Recrystallization is not really necessary, since the solid obtained by evaporating the aqueous solution after neutralization with sodium hydroxide appears to be pure. The loss in hydrazinolysis is partly apparent, since the granules of polymer contain 5–10% of lipid (Macrae & Wilkinson, 1958). Nevertheless, the recovery was below that expected. If free β -hydroxybutyric acid were produced during the depolymerization, e.g. from the $-CO_2H$ terminal groups of the chains, it, or its hydrazine salt, might have been lost during crystallization from propanol. This possibility has not been investigated. There might also have been a loss through some chemical reaction associated with the evolution of gas during depolymerization. It is presumed that this gas is nitrogen, arising from oxidation of hydrazine, although it is not immediately apparent what the oxidant can be.

It is of interest that poly- β -hydroxybutyrate should contain the D(–) isomer of the acid, if it is

formed by condensation of β -hydroxybutyryl residues from the CoA derivative. It has been shown that in mammalian liver the metabolically active form is L(+)- β -hydroxybutyryl-CoA (Lehninger & Greville, 1953; Lynen & Decker, 1957).

SUMMARY

1. Pure D(–)- β -hydroxybutyric acid has been prepared by the hydrazinolysis of bacterial poly- β -hydroxybutyric acid.

2. The main product of hydrazinolysis, D(–)- β -hydroxybutyric hydrazide, was obtained in crystalline form and was converted into the free acid by hydrolysis with hydrochloric acid at 105° for 3 hr.

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The Behaviour of the *Bz*-Methylindoles as Substrates and Inhibitors for *Neurospora crassa* Tryptophan Synthase

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The present investigation was undertaken to gain further knowledge of the nature of enzyme–substrate interaction in tryptophan synthesis and complement a previous investigation of the degradation of certain *Bz*-substituted tryptophans by *Escherichia coli* tryptophanase (Hall, Leeson, Rydon & Tweddle, 1960).

Tryptophan synthase [L-serine hydro-lyase (adding indole), EC 4.2.1.20] was first recognized by Tatum & Bonner (1943, 1944), who demonstrated the ability of extracts of *Neurospora crassa* to catalyse a condensation between indole and L-serine to yield L-tryptophan. The requirement of the enzyme for pyridoxal phosphate was demonstrated

by Umbreit, Wood & Gunsalus (1946). Yanofsky (1952) and Myers & Adelberg (1954) demonstrated a stoichiometric equivalence between the amounts of indole consumed and of tryptophan produced in the reaction. More recent work (Yanofsky & Rachmeler, 1958; De Moss, Imai & Bonner, 1958; Yanofsky, 1959) has shown that tryptophan synthase is a remarkable enzyme in that, in addition to catalysing the condensation of indole and serine, it can also effect two other reactions, namely the reversible breakdown of indol-3-ylglycerol phosphate to indole and triose phosphate, and a transfer process between indol-3-ylglycerol phosphate and serine yielding tryptophan and triose phosphate. It now seems likely that free indole is not a direct intermediate in the normal synthesis of tryptophan in *E. coli* and *N. crassa* (Yanofsky, 1960).

Yanofsky (1960) has reviewed many aspects of the tryptophan-synthase system and has drawn attention to the fact that, whereas the *E. coli* enzyme system can be resolved into two components, the *N. crassa* enzyme behaves as a single protein during fractionation.

A little information is available relating to the affinity of tryptophan synthase for the *Bz*-methylindoles. Yanofsky (1955) has reported that 6-methylindole is attacked by the enzyme but at a lower rate than is obtained with an equimolar concentration of indole. Growth studies by Fildes & Rydon (1947) demonstrated the ability of the four *Bz*-methylindoles to serve as inhibitors of the utilization of indole by certain strains of *Salmonella typhi*.

MATERIALS AND METHODS

Chemicals. The 4-, 5- and 6-methylindoles were prepared according to the methods of Marion & Oldfield (1947) and of Rydon (1948). 7-Methylindole was prepared by cyclization of *N*-formyl-2,6-dimethylaniline in the presence of potassium *tert*-butoxide according to Tyson (1941). The 4-, 5-, 6- and 7-methyltryptophans became available through the work of Hall *et al.* (1960).

Pyridoxal phosphate was a gift from Parke, Davis and Co.

Organism. *Neurospora crassa* (wild-type strain SY 7A), kindly given by Professor E. L. Tatum, was used. The organism was maintained on slants of a medium containing (w/v): Difco yeast extract (0.5%), Evans peptone (0.5%), maltose (4%) and agar (2%). Reserve cultures were prepared by freeze-drying spores in the presence of skimmed milk.

Determination of indole and methylindoles. In certain experiments the amounts of these compounds utilized in the tryptophan-synthase reaction were estimated by either the xanthidrol method (Hogness & Mitchell, 1954) or by use of the Ehrlich reaction (Hall *et al.* 1960). In both procedures extinction was measured in a 1 cm. cell by reading against water in a Hilger Spekker Photoelectric Absorptiometer with an Ilford Spectrum yellow-green filter no. 605 (wavelength of peak transmission 550 m μ).

Determination of tryptophan and methyltryptophans.

Samples (10 ml.) from reaction mixtures which had been treated with 0.2 ml. of 50% (w/v) trichloroacetic acid were centrifuged for 20 min. at 2000g. The supernatants were then extracted with pure toluene (5 ml.) to remove unchanged indole or methylindole, and the tryptophan content of an 8 ml. portion of the aqueous phase was assayed by 'procedure A' of Spies & Chambers (1948). Extinction was measured in a 4 cm. cell against a water blank with an Ilford Spectrum Orange filter no. 607 (wavelength of peak transmission 600 m μ). For accurate estimation a standard curve was prepared for each experiment. As tryptophan and the methyltryptophans gave linear relationships between extinction and concentration it was possible to use the method of least squares to calculate the equations of standard curves; the concentration term was taken to be the independent variable.

Cultivation of the mould. 'Medium no. 3' of Fries (1938), as modified by Ryan, Beadle & Tatum (1943) and supplemented with DL-serine (0.1 mM), was distributed in 330 ml. amounts in Glaxo flasks (Clayton, Hems, Robinson, Andrews & Hunwicke, 1944). The flasks were steamed for 15 min. at atmospheric pressure and then autoclaved for 15 min. at 15 lb./in.² The medium was inoculated with spores of *N. crassa* SY 7A, from a young stock culture, and incubated for 3 days at 25° with mechanical agitation (horizontal reciprocation at 55 strokes/min.). The mycelium was then harvested by filtration through surgical gauze and washed several times with water. Surplus moisture was expelled by squeezing. The mycelium was stored in polythene bags at -28°.

Preparation of enzyme extracts. The method employed was a modification of the one described by Hogness & Mitchell (1954), designed to avoid the necessity for low-temperature centrifuging.

Frozen mycelium (30 g.) was triturated by hand for 7 min., in a mortar surrounded by ice, with 10 g. of washed carborundum powder (180 grade, Carborundum Co. Ltd., Trafford Park, Manchester) and 30 ml. of 0.1M-potassium phosphate buffer, (pH 7.6), containing 70 mg. of DL-serine. The mixture was centrifuged for 5 min. at 6400g to yield a turbid supernatant which was decanted into a 100 ml. Erlenmeyer flask and then heated in a water bath, controlled at 75°, for 6 min., the temperature of the solution never being allowed to exceed 54°. If appreciable turbidity developed at this stage, the extract was again centrifuged for 5 min. at 6400g and the residue discarded. Ammonium sulphate (18.7%, w/v) was then added slowly, with stirring, to the ice-cold extract and the mixture kept at 0° for 40 min. The resulting precipitate was collected by centrifuging for 10 min. at 6400g, washed with 20 ml. of 0.1M-potassium phosphate buffer (pH 7.6) at 0° containing ammonium sulphate (19.1%, w/v) and finally extracted by suspending in 15 ml. of 0.1M-phosphate buffer at 0°. Centrifuging for 10 min. at 6400g yielded a partially purified enzyme extract which was stored at -28°.

Optimum conditions. The conversion of 7-methylindole and L-serine into 7-methyltryptophan by enzyme extracts was measured at pH 7.5, 7.6, 7.7, 7.8 and 7.9 in 0.1M-potassium phosphate buffer; the concentrations of 7-methylindole and of DL-serine were 0.33 mM and 0.16M respectively. No significant variation in activity was noted over this pH range, and, as Hogness & Mitchell (1954) had reported pH 7.6 to be optimum for indole, this value was chosen for all the kinetic experiments.

No adverse effect of phosphate buffer on either enzyme activity or on the colorimetric estimation of 7-methyltryptophan could be demonstrated in media (pH 7.6) containing phosphate buffer at concentrations of 0.05, 0.1, 0.2 and 0.4M. The choice of 0.1M for buffer strength was thus believed to be satisfactory. All reactions were carried out at $37 \pm 0.01^\circ$.

Determination of kinetic constants. Preliminary experiments showed that enzyme activity was enhanced by preincubation of the extracts with pyridoxal phosphate (0.2 mM) at 37° ; the extent of activation, however, varied with the batch of enzyme used. Activation was complete in 10 min. and preincubation at 37° for 10 min. was adopted as a standard procedure.

Enzyme-pyridoxal phosphate mixture at 37° was added at 1 min. intervals to solutions of the appropriate indole (or mixture of indoles) and DL-serine in potassium phosphate buffer distributed in test tubes (1.6 cm. $\times$ 15.5 cm.). The concentrations of pyridoxal phosphate and DL-serine in the reaction mixture were 0.04 mM and 0.16M re-

spectively; the volume of solution was 10 ml. Three replicate tubes were used for each concentration of indole or methylindole. The addition of enzyme to a set of tubes was performed in a random manner to minimize the effect of possible variations in enzyme activity with time. After incubation for an accurately timed period, enzyme activity was halted by the addition of 0.2 ml. of 50% (w/v) trichloroacetic acid. Values of K_m were evaluated by a procedure based on the integrated form of the Michaelis-Menten equation:

$$[E]k_{+2}t = K_m \ln \left\{ \frac{[S]}{[S] - [P]} \right\} + [P]$$

where $[S]$ is the initial substrate concentration, $[P]$ is the concentration of product after time t , $[E]$ is the concentration of enzyme and k_{+2} is the first-order velocity constant for the breakdown of the enzyme-substrate complex. The terms $\ln \{[S]/([S] - [P])\}$ and $[P]$ were calculated for each concentration of indole tested and plotted against each other. Intercepts were calculated by the method of least squares; K_m is the slope of the line. It was possible to obtain two values of K_m from a given set of data by treating $\ln \{[S]/([S] - [P])\}$ and $[P]$, in turn, as independent variables. The criterion of a good set of data is that the two calculated values of K_m should agree closely.

Values of K_i for those methylindoles which were found to be poor substrates were calculated in a similar manner from the equation:

$$[E]k_{+2}t = \ln \left\{ \frac{[S]}{[S] - [P]} \right\} K_m \left(1 + \frac{[I]}{K_i} \right) + [P]$$

where $[I]$ is the inhibitor concentration used. The previously determined mean value of K_m for indole was substituted in the equation. To avoid a multiplicity of K_i values from one experiment, each statistical analysis was performed with the product term as the independent variable.

The complete data for a typical substrate (indole) and for an inhibitor (5-methylindole) are given in Tables 1 and 2 respectively; the complete data for all the substrates and inhibitors are given by Lea (1958).

RESULTS

Stoichiometry of the conversion of 4-methylindole into 4-methyltryptophan. 4-Methylindole (0.138 mM) was allowed to react with serine in the presence of enzyme for 60 min., and 4-methylindole and 4-methyltryptophan were estimated. A disappearance of 0.25 μ mole of 4-methylindole coincided with the accumulation of 0.22 μ mole of 4-methyltryptophan.

Enzyme-substrate and enzyme-inhibitor dissociation constants. The results (Table 3) are expressed in terms of the Michaelis constant, K_m (i.e. k_{-1}/k_{+1}), for the dissociation of the enzyme-substrate complex, and of the inhibitor constant, K_i (k_{-1}/k_{+1}), for the dissociation of the enzyme-inhibitor complex according to the Michaelis-Menten formulae:

Table 1. *Determination of K_m for indole*

The molar concentrations of substrate, L-tryptophan and the Michaelis constant are denoted by $[S]$, $[P]$ and K_m respectively.

Expt. no.	$10^4[S]$	$10^5[P]$	$10^5 K_m$	
			[P] as independent variable	$\ln \{[S]/([S] - [P])\}$ as independent variable
1	3	1.71	—	—
	1.2	1.53	—	—
	0.6	1.33	—	—
	0.3	1.14	1.4	1.1
2	3	1.64	—	—
	1.2	1.45	—	—
	0.6	1.36	—	—
	0.3	1.14	1.2	1.1
3	3	1.47	—	—
	1.2	1.30	—	—
	0.6	1.20	—	—
	0.3	1.05	1.1	1.0

Table 2. *Determination of K_i for 5-methylindole*

The molar concentrations of indole, 5-methylindole, L-tryptophan and the inhibitor constant are represented by $[S]$, $[I]$, $[P]$ and K_i respectively, and are calculated on the basis that K_m for indole is 1.2×10^{-5} M.

Expt. no.	$10^4[S]$	$10^4[I]$	$10^5[P]$	$10^5 K_i$
1	4	0	3.06	—
	4	5.9	2.48	8
2	4	5.9	2.31	—
	4	2.5	2.61	—
	2	5.9	1.99	—
	2	2.5	2.34	—
	1	5.9	1.52	12
	1	2.5	1.95	9
3	4	5.9	2.98	—
	4	2.5	3.48	—
	2	5.9	2.37	—
	2	2.5	3.01	—
	1	5.9	1.82	8.6
	1	2.5	2.50	8.0

All four *Bz*-methylindoles acted as substrates for *N. crassa* tryptophan synthase, being converted into the corresponding *Bz*-methyltryptophans, although very much more slowly than indole was converted into tryptophan (Table 4). Of the four, the 7- and 4-methyl compounds were the best substrates; the 6- and 5-methyl compounds were much less effective as substrates and indeed were attacked too slowly to enable K_m values to be determined.

The 5- and 6-methyl compounds showed pronounced inhibitory activity towards tryptophan synthase, and K_i has been determined for them; 4-methylindole also showed slight activity as an inhibitor, but this was insufficient for the evaluation of K_i ; 7-methylindole lacked demonstrable inhibitory activity.

DISCUSSION

The value of K_m for indole (0.012 mM), obtained in the present work, with partially purified tryptophan synthase, is appreciably lower than the values reported by Hogness & Mitchell (1954) (0.023 and 0.028 mM) and by Rachmeler & Yanofsky (1961) (0.022 mM). Although these workers did not describe their methods of calculation, they all relied on determinations of indole utilized, whereas we determined the amount of tryptophan produced.

Table 3. *Enzyme-substrate and enzyme-inhibitor dissociation constants*

The dissociation constants (M) are expressed as mean values $\pm$ s.e. with the number of determinations in parentheses.

Substituent	$10^5 K_m$	$10^5 K_i$
H	1.2 ± 0.06 (3)	—
4-Me	32.0 ± 3.6 (3)	—
5-Me	—	9.5 ± 0.83 (5)
6-Me	—	11 ± 2.6 (4)
7-Me	9.0 ± 0.6 (4)	—

Since, in determining K_m , the degree of conversion of substrate into product should be kept as low as possible, it follows that, even when the analytical methods available for the determination of substrate and product are of comparable accuracy, more exact values of kinetic constants should be obtained with direct determination of small amounts of product formed than by determination, by difference, of small amounts of substrate utilized.

Our work was undertaken in the hope that it would provide a more quantitative picture of the observations of Fildes & Rydon (1947), who showed that the four *Bz*-methylindoles acted as competitive inhibitors of an indole-requiring strain of *Salmonella typhi*, with inhibitory activities in the decreasing order: 4-, 6-, 7-, 5-Me. In the event this hope was not realized, since, at least with *N. crassa* tryptophan synthase, all four *Bz*-indoles showed substrate activity, only two of them (the 5- and 6-methyl compounds) being sufficiently powerful inhibitors to enable K_i to be determined.

Consideration of the dissociation constants in Table 3 shows clearly that the introduction of a methyl group into the benzene ring of indole decreases the affinity for tryptophan synthase. Since, however, two of the four measured dissociation constants for the *Bz*-methylindoles relate to substrates and two to inhibitors it would seem unwise to attempt any detailed theoretical analysis of the results, such as was possible for the experiments of Fildes & Rydon (1947), who were dealing with a group of inhibitors, and those of Hall *et al.* (1960), who were dealing with a group of substrates. It is, however, reasonable to suggest that the low affinity of the synthase for 4-methylindole has a stereochemical origin. Inspection of models shows clearly that the 4-methyl group causes considerable shielding of the 3-position; this would inhibit approach by serine, or a modified serine, to form the complete apoenzyme-coenzyme-serine-indole complex which must be involved in synthase action.

Table 4. *Comparison of the utilization and transformation of indole and the Bz-methylindoles*

Expt. no.	Substituent	$10^5 \times$ initial concn. of indole (M)	Reaction time (min.)	Indole utilized (%)	Methyltryptophan formed (%)
1	H	4	15	43.0	
	4-Me	4	60	14.7	
	5-Me	4	60	4.1	
	6-Me	4	60	6.7	
	7-Me	4	60	24.0	
2	4-Me	12	30	11.4	
	5-Me	60	30	0.7	
	6-Me	12	30	3.3	
	7-Me	30	30	14.7	

SUMMARY

1. A partially purified preparation of tryptophan synthase from *Neurospora crassa* has been shown to catalyse the conversion of all four *Bz*-methylindoles into the corresponding methyltryptophans.

2. The four *Bz*-methylindoles are much less effective substrates than indole itself, the 7- and 4-methyl compounds being the most effective, followed by the 6- and 5-methyl compounds, in descending order of effectiveness.

3. The equilibrium constants, K_m , for the dissociation of the enzyme-substrate complexes with indole and 4- and 7-methylindoles have been evaluated.

4. The low affinity of the enzyme for 4-methylindole is ascribed to shielding of the 3-position by the 4-methyl group.

5. The 5- and 6-methylindoles inhibit the conversion of indole into tryptophan by the enzyme and the equilibrium constants, K_i , for the dissociation of the enzyme-inhibitor complexes have been evaluated.

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Further Observations on the Intracellular Location and Mechanism of Action of Liver Enzymes Catalysing the Synthesis of L-Ascorbic Acid

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Evidence has been presented that the enzyme systems catalysing the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid in animal tissues reside in the microsomal fractions of liver homogenates of rat and goat, and kidney homogenate of the chick (Burns, Peyser & Moltz, 1956; Chatterjee, Ghosh, Ghosh & Guha, 1958*b*; Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1959*a*, 1960*a*; Suzuki, Mano & Shimazono, 1960). It was also reported that the enzymes can convert

only the lactone forms of the precursors, namely D-glucuronolactone and L-gulonolactone, but not the corresponding free acids: the sodium salts of the corresponding free acids can be converted into L-ascorbic acid only after lactonization by incubating them with an aldonolactonase preparation from the soluble supernatant (Chatterjee *et al.* 1958*a*, 1960*a*).

However, Isherwood, Mapson & Chen (1960) reported that, not only the microsomes, but also the mitochondrial fraction of rat-liver homogenate can convert L-gulonolactone into L-ascorbic acid,

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