The Degradation of some *Bz*-Substituted Tryptophans by *Escherichia coli* Tryptophanase

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The present paper is the second in a series designed to correlate substrate structure with the kinetic constants of enzyme-catalysed reactions and thus throw light on the nature of the attachment of the substrate to the enzyme.

Since the preparation of the first paper (Nath & Rydon, 1954), which describes the influence of structure on the hydrolysis of substituted phenyl-β-D-glucosides by emulsin, investigations of a similar nature have been reported by Gawron, Grelecki & Duggan (1953) on the hydrolysis of substituted phenyl acetates by wheat-germ lipase and by Dodgson, Spencer & Williams (1956) on the hydrolysis of substituted phenyl sulphates by the arylsulphatase of *Alcaligenes metalcaligenes*.

Selection of the tryptophanase system for the current study stems from the observation of Anderson (1945) that 5-methyltryptophan is inhibitory to the growth of *Escherichia coli* and from the demonstration by Fildes & Rydon (1947) that the four *Bz*-methyltryptophans are decreasingly effective as inhibitors of *Salmonella typhi* in the order 4-methyl-, 5-methyl-, 6-methyl- and 7-methyl-tryptophan and are also competitive with tryptophan. The growth-inhibitory properties of the methyltryptophans have been examined rather more recently by other workers (Marshall & Woods, 1952; Akiba & Arai, 1951; Beerstecher, 1954; Trudinger & Cohen, 1956).

The formation of 5-methylindole from 5-methyltryptophan in the presence of a suspension of viable cells of *E. coli* was studied by Beerstecher & Edmonds (1951). According to these workers 5-methyltryptophan is degraded only in the presence of small amounts of tryptophan or indole. The reaction was considered, therefore, to be autocatalytic in the presence of tryptophan. While the present work was in progress Trudinger & Cohen (1956) demonstrated the formation of 4-methylindole from 4-methyltryptophan by tryptophanase-containing extracts of *E. coli* cells and cited evidence in favour of the view that the decompositions of tryptophan and of 4-methyltryptophan are catalysed by the same enzyme system. Mention should also be made of preliminary observations on the action of tryptophanase on substituted methyltryptophans by Dr D. Herbert (see Fildes & Rydon, 1947) and by R. B. Beechey & F. C. Happold (personal communication). The order of efficacy of the *Bz*-methyltryptophans as substrates for tryptophanase was given by Herbert (in decreasing order) as 4-methyl-, 6-methyl-, 5-methyl- and 7-methyl-tryptophan. Beechey & Happold found the same order of reactivity at low concentrations of substrate, but at higher concentrations the positions in the series of 4-methyl- and 6-methyltryptophans were interchanged.

We describe below the determination of the equilibrium (affinity) constant, K_a ($= k_1/k_2$), which controls the formation of the enzyme–substrate complex, and the first-order velocity constant, k_3, for the breakdown of this complex into enzyme and products in the degradation of L-tryptophan and eight of its substitution products (4-, 5-, 6- and 7-methyl- and 4-, 5-, 6- and 7-chloro-) by cell-free tryptophanase and by washed cell suspensions of *E. coli*.

MATERIALS AND METHODS

Preparation of indoles and tryptophans

The 4-, 5- and 6-methylindoles were prepared according to the methods of Marion & Oldfield (1947) and of Rydon (1948*a*). 7-Methylindole was a commercial product. The 4-, 5-, 6- and 7-methyltryptophans were prepared in the first instance according to Rydon (1948*a*), further quantities being prepared by the procedures of Snyder, Beilfuss & Williams (1953). The four *Bz*-chloroindoles and the derived *Bz*-chlorotryptophans were made available by the work of Rydon & Tweddle (1955).

Analytical methods

Indole and Bz-*substituted indoles.* The quantities of these compounds produced in the degradations of tryptophan and the substituted tryptophans respectively were estimated by means of the Ehrlich reaction. The rosindoles produced by interaction of the indoles with *p*-dimethylaminobenzaldehyde were estimated colorimetrically with a Spekker photoelectric absorptiometer and an Ilford Spectrum yellow–green filter no. 605 (wavelength of peak transmission 550 mμ).

The procedure used required the addition of 5 ml. of *p*-dimethylaminobenzaldehyde reagent, prepared by dissolving 4 g. of *p*-dimethylaminobenzaldehyde in a mixture of 380 ml. of ethanol and 80 ml. of conc. hydrochloric acid (Cunningham, 1947), to the reaction mixture (5 ml.). After

the colour had been allowed to develop to its maximum value, for a previously determined time which was different for different indoles and for different experimental conditions, the extinction was read, usually in a 1 cm. cell, against a blank treated in the same manner as the reaction samples. It was advantageous to read the colours produced by the chloroindoles in 2 cm. cells. At the time of each experiment reference curves were constructed for the appropriate indoles in the range up to 0·075 mM in the presence of trichloroacetic acid and enzyme extract or washed cell suspension.

Total nitrogen. The total nitrogen content of enzyme extracts and of washed cell suspensions was determined by the Kjeldahl procedure, with selenium as catalyst. The ammonia produced was liberated by adding conc. aq. sodium hydroxide, steam-distilled into 2% boric acid solution and finally titrated with 0·01 N-hydrochloric acid in the presence of the mixed indicator recommended by Klein (1947).

Preparation of enzyme extracts

(1) *From acetone-dried cells of* E. coli. Cultures (6 hr.) of *E. coli* in a medium consisting of tryptic digest of casein (Davis, 1939) and DL-tryptophan (25 mg./l.) at pH 7·4 were used to inoculate 300 ml. portions of the same medium solidified with agar (4½%, w/v) and contained in enamelled-metal trays (surface area, 940 sq.cm.) fitted with aluminium covers. After incubation at 37° for 18 hr. the cells were harvested by washing from the agar surfaces with sterile aq. 0·9% sodium chloride (about 20 ml./tray). The cells were collected by centrifuging, suspended in water and then acetone-dried according to the method of Dawes & Happold (1949); the average yield of acetone-dried cells was 320 mg./tray. A supply of several grams of acetone-dried cells was prepared and stored over calcium chloride until required for use. Tryptophanase was extracted from acetone-dried cells with 0·8 M-potassium chloride containing pyridoxal phosphate (100 μg./ml.) under the conditions recommended by Dolby, Hall & Happold (1952). The incorporation of pyridoxal phosphate into the potassium chloride solution was shown to lead to extracts of considerably higher activity than those prepared in its absence, owing to stabilization of the enzyme against thermal inactivation. Moreover, in an investigation such as the present one, it is clearly desirable that the apoenzyme should not only be protected so far as possible against thermal inactivation, but also be fully saturated with coenzyme.

Enzyme extracts were usually prepared on the day before use and stored at −20°. The frozen extracts were allowed to thaw when required, care being taken to ensure that the temperature never exceeded 2°. Extracts were then diluted with 0·8 M-potassium chloride at 0° to give the required activity and maintained at this temperature during the course of an experiment. On several occasions the activities of diluted extracts were shown to remain constant for several hours at 0°.

The activities of original extracts were assayed and expressed in arbitrary units (μmoles) of indole formed in 4 min. at 37° from 2·5 μmoles of L-tryptophan, 0·5 mM in 0·16 M-phosphate buffer (pH 7·4), by the action of 0·1 ml. of extract (mean activity, 0·046 ± 0·001 for 13 extracts; mean nitrogen content, 0·40 mg./ml.).

(2) *From washed cells of* E. coli. After cultivation of *E. coli* in liquid casein digest–tryptophan medium (300 ml.) for 18 hr. at 37° the cells were collected and washed as described below. The cells were ground with Ballotini beads no. 11 (English Glass Co. Ltd., Leicester) for about 5 min. at room temperature, by using a glass mortar and pestle; 0·8 M-potassium chloride (20 ml.) was then added and extraction of the enzyme achieved by standing for 2 hr. at 37°. The suspension was finally centrifuged to yield a supernatant with considerable tryptophanase activity (N content 0·13 mg./ml.).

Preparation of washed cell suspensions. Casein digest–tryptophan medium (15 ml.) was inoculated with a 6-day culture of *E. coli* and incubated for 24 hr. at 18°. The same medium (50 ml.) was then inoculated with 0·01 ml. of the 24 hr. culture. After incubation for 18 hr. at 37° the cells were collected by centrifuging, suspended in saline and re-centrifuged. The process was repeated until indole could no longer be detected in the washings with *p*-dimethylaminobenzaldehyde reagent. The cells were finally suspended in a small volume of aq. 0·9% sodium chloride to yield a stock suspension which was further diluted to give suspensions of activity suitable for use in kinetic experiments. Fresh stock suspensions were prepared for each experiment by repeating the subculturing programme.

The determination of the extinctions and nitrogen contents of a series of cell suspensions facilitated the construction of a reference curve relating the extinctions and total nitrogen content (mg. of N/ml.) of cell suspensions. The extinctions were measured with a Spekker photoelectric absorptiometer by using the neutral-grey filter no. H 508.

Optimum conditions. The extent of degradation of tryptophan and the eight *Bz*-substituted tryptophans in 15 min. at 37° by a washed cell suspension of *E. coli* was measured at pH 7·2, 7·3, 7·4, 7·5 and 7·6; the concentrations of phosphate buffer and of the L-tryptophan were 0·14 M and 10^{-4} M respectively. All the tryptophans showed maximum conversion into the corresponding indoles at pH 7·4. On this basis all experiments were performed at pH 7·4; Dawes & Happold (1949) give the optimum pH of tryptophanase as 7·5.

Experiments on the formation of indole from L-tryptophan showed that, within the concentration range 0·08–0·24 M, phosphate buffer has no effect on the rate of indole formation.

All degradations were carried out at 37 ± 0·01°.

Determination of kinetic constants for cell-free tryptophanase. Portions (4 ml.) of solutions of the tryptophan in 0·2 M-phosphate buffer, pH 7·4 (Clark & Lubs; cf. Vogel, 1947), were distributed in duplicate in rimless test tubes (15·5 cm. × 1·6 cm.). The tubes were preheated at 37° for 10 min. and 1 ml. of diluted tryptophanase extract at 0° was added. After incubation for a time which was constant in any particular experiment, and sufficient to give a suitable yield of the indole, enzyme activity was halted by the addition of 0·1 ml. of 50% trichloroacetic acid solution for tryptophan and the methyltryptophans, and analysis for the indole undertaken immediately. In reactions with the chlorotryptophans the addition of 5 ml. of *p*-dimethylaminobenzaldehyde reagent served to inactivate the enzyme. A blank prepared by adding enzyme extract (1 ml.) to buffer (4 ml.) was always included in experiments, since correction was necessary for small amounts of protein

Table 1. *Degradation of* 6-*methyltryptophan by* Escherichia coli *tryptophanase at* 37° (pH 7·4)

e'_0 = Enzyme concentration (g. of acetone-dried cells/l.).
s_0 = Substrate concentration (moles of L-enantiomorph/l.).
t = Reaction time (min.).
x = 6-Methylindole formed (in 5 ml.) (moles).
v = Reaction velocity (moles/l./min.).
$V_{max.}$ = Limiting velocity (moles/l./min.).
K_a = Equilibrium constant (l./mole).
k'_3 = First-order velocity constant for decomposition of enzyme-substrate complex (min.$^{-1}$).

Enzyme prep.	e'_0	t	$10^4 s_0$	$10^6 x$	$10^6 v$	$10^6 V_{max.}$	K_a	$10^5 k'_3$
M_1	0·20	11	2·0	0·028 0·028	0·51 0·51	3·62	810	1·81
			3·0	0·0385 0·037	0·70 0·67			
			4·0	0·0505 0·0473	0·92 0·86			
			5·0	0·0565 0·0595	1·03 1·08			
			6·0	0·0645 0·0672	1·17 1·22			
M_2	0·20	14	2·0	0·0315 0·0315	0·45 0·45	3·14	850	1·57
			3·0	0·046 0·046	0·66 0·66			
			4·0	0·056 0·056	0·80 0·80			
			5·0	0·062 0·062	0·89 0·89			
			6·0	0·077 0·0745	1·10 1·06			
M_3	0·20	14	2·0	0·034 0·034	0·49 0·49	2·62	1130	1 31
			3·0	0·0455 0·0455	0·65 0·65			
			4·0	0·059 0·061	0·84 0·87			
			5·0	0·0685 0·0655	0·98 0·98			
			6·0	0·070 0·0745	1·00 1·06			
M_4	0·20	14	2·0	0·033 0·033	0·47 0·47	2·88	970	1·44
			3·0	0·043 0·043	0·61 0·61			
			4·0	0·0565 0·0565	0·81 0·81			
			5·0	0·0655 0·0655	0·94 0·94			
			6·0	0·076 0·076	1·09 1·09			
						Mean values	940	1·53
						S.E.	±72	±0·11

which were usually precipitated by the addition of trichloroacetic acid or *p*-dimethylaminobenzaldehyde reagents.

The values of K_a and $V_{max.}$ were calculated from the linear relationship between $1/v$ and $1/s_0$ (Lineweaver & Burk, 1934; Dixon, 1953). The slopes of the best straight lines relating these data were calculated by the method of least squares so applied as to include individual replicate values of v. The accuracy of each K_a value was then tested by analysis of variance and the standard deviation evaluated.

Values of k'_3, which is the required first-order velocity constant k_3 multiplied by an unknown constant factor, were evaluated from the calculated values of $V_{max.}$

($= k_3' e_0'$); e_0' was expressed in arbitrary units of g. of acetone-dried cells/l. of enzyme extract, these units being used in preference to units involving the nitrogen content of the enzyme extracts because of the lack of correlation between nitrogen content and activity of extracts. The complete data for one typical substrate (6-methyltryptophan) are given in Table 1; the complete data for all the substrates are given by Leeson (1956).

Determination of kinetic constants for washed cells of E. coli. To test tubes containing the tryptophan in 4 ml. of 0·2 M-phosphate buffer (pH 7·4), preheated at 37°, were added 1 ml. portions of a cell suspension of measured extinction. After incubation for the appropriate period, enzyme activity was inhibited by the addition of 0·1 ml. of 50% trichloroacetic acid solution. Before analysis for indole content, the reaction mixtures were clarified by centrifuging for 6 min. at 4000 rev./min. Measurements were made, in duplicate, on three concentrations of the tryptophan with reaction times of 3, 6, 9 and 12 min. The experiment was repeated with a further two or three cell suspensions differing in extinction from the first suspension.

The values of K_a were evaluated from values of K_m ($K_a = 1/K_m$), which were themselves evaluated by the method of Veibel & Lillelund (1940), which utilizes the plot of $1/k_0$ against s_0. The overall velocity constants (k_0) required in this method were calculated from the slope of the linear plot (drawn by the method of least squares) of the decadic logarithm of the concentration of unchanged substrate against time (min.). The values of k_3' were calculated from the slope of the best straight line for the plot of $1/k_0$ against s_0 by using the expression $1/k_0 = (K_m + s_0)/k_3' e_0'$ (cf. Nath & Rydon, 1954), e_0' being the concentration of bacterial nitrogen (g.atom of N/l.). Three values of K_a and k_3' were obtained for each tryptophan. The complete data

Table 2. *Degradation of* 6-*methyltryptophan by* Escherichia coli *at* 37° (pH 7·4)

e_0' = Enzyme concentration (g.atoms of bacterial N/l.).
s_0 = Substrate concentration (moles of L-enantiomorph/l.).
t = Time (min.).
x = 6-Methylindole formed (in 5 ml.) (moles).
k_0 = Overall velocity constant (min.$^{-1}$).
k_3' = First-order velocity constant for decomposition of enzyme–substrate complex (min.$^{-1}$).
K_a = Equilibrium constant (l./mole).

$10^5 e_0'$	$10^5 s_0$	t	$10^6 x$	k_0	$10^5 k_3'$	K_a
2·5	2·0	6	0·005	0·0082	1 390	50 000
		9	0·008			
		12	0·009			
	5·0	6	0·007	0·0046		
		9	0·009			
		12	0·014			
	10·0	3	0·004	0·0027		
		6	0·008			
		9	0·012			
		12	0·023			
5·0	2·0	3	0·002	0·0130	1 060	43 000
		6	0·006			
		9	0·011			
		12	0·016			
	5·0	3	0·005	0·0070		
		6	0·010			
		9	0·015			
		12	0·023			
	10·0	3	0·007	0·0044		
		6	0·009			
		9	0·019			
		12	0·026			
10·5	2·0	3	0·003	0·0332	1 350	41 000
		6	0·011			
		9	0·021			
		12	0·026			
	5·0	3	0·004	0·0182		
		6	0·026			
		9	0·034			
		12	0·047			
	10·0	3	0·008	0·0115		
		6	0·037			
		9	0·045			
		12	0·069			
				Mean values	1 270	45 000
				±S.E.	±100	±2 700

for one typical substrate (6-methyltryptophan) are given in Table 2; the complete data for all the substrates are given by Tweddle (1957).

RESULTS

The results of experiments with both the cell-free enzyme and with washed cells of *E. coli* are given in Table 3; they are expressed in terms of the affinity constant, $K_a(= k_1/k_2)$, for the formation of the enzyme–substrate complex and of the first-order velocity constant, k_3, for the decomposition of the complex according to the Michaelis–Menten formulation:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + P.$$

The K_a values recorded are all calculated in terms of the L-tryptophans, the D isomers being assumed to be inert. For tryptophan itself, the work of Woods (1935) with washed cell suspensions, and of Gooder & Happold (1954) with enzyme preparations, has demonstrated the inability of tryptophanase to attack D-tryptophan. Furthermore, according to Gooder & Happold, D-tryptophan does not inhibit the formation of indole from L-tryptophan and therefore is also inactive as an inhibitor. The inert nature of D-tryptophan under the conditions of the present study was further confirmed experimentally in the course of the present work; indole formation from L- and DL-tryptophan equimolar with respect to the L isomer was identical with both cell-free tryptophanase and washed cell suspensions.

At an early stage of the work it became apparent that a considerable difference existed between the K_a values for the degradation of L-tryptophan by cell-free enzyme extracts and by washed cell suspensions. It was therefore desirable to ensure that the treatment of washed cells of *E. coli* with acetone and certain other organic solvents does not adversely affect the affinity of tryptophanase for its substrate. In consequence, determinations of K_a for L-tryptophan were made on an enzyme extract prepared according to procedure (2), which does not involve treatment of the cells with acetone. The K_a values so obtained (2150 and 2350 l./mole) agree reasonably well with those (1920 ± 120) determined by using extracts of acetone-treated cells (procedure 1). Although the extract prepared by procedure (2) was considerably more active than those prepared by procedure (1) and had a considerably lower nitrogen content, it was rather unstable and hence not convenient for routine use. The lower stability of this preparation may be accounted for by its greater purity, since Dawes & Happold (1949) have found that the stability of the enzyme decreases on purification.

DISCUSSION

The K_a obtained for L-tryptophan with the cell-free enzyme preparation (1920 l./mole) is of the same order as that (2400 l./mole) obtained by Dawes & Happold (1949) with partially purified tryptophanase. It appears unlikely, therefore, that the K_a values obtained for the eight substituted tryptophans with our impure tryptophanase preparation would differ greatly from those which would be obtained with a purified enzyme preparation.

In correlating the kinetic constants for the breakdown of *Bz*-substituted tryptophans with the substituent constants, σ (Hammett, 1940), the latter can be chosen in two ways. If the main effect is on N-1 of the indole nucleus, then 4- and 6-substituents are to be regarded as *meta* substituents, 5-substituents as *para* and 7-substituents as

Table 3. *Kinetic constants for degradation of* Bz-*substituted tryptophans*

K_a = Equilibrium constant for degradation at 37° in 0·16 M-phosphate buffer (pH 7·4) by (i) cell-free tryptophanase and (ii) washed cells of *E. coli* (l./mole).
k_3' = First-order velocity constant for decomposition of enzyme–substrate complex under above conditions (min.$^{-1}$).
σ = Substituent constant. (Values for *meta*- and *para*-substituents are from Hammett (1940); for *ortho* substituents, from Mamalis & Rydon (1955).
Kinetic constants are mean values ± S.E., followed by the number of determinations in parentheses.

	(i) Cell-free enzyme		(ii) Washed cells		
Substituent	K_a	$10^5 k_3'$	K_a	$10^5 k_3'$	σ
H	1 920 ± 120 (6)	2·17 ± 0·17 (6)	167 000 ± 10 000 (10)	3 770 ± 200 (10)	0·000
4-Me	350 ± 25 (3)	1·22 ± 0·11 (3)	71 000 ± 7 000 (3)	1 150 ± 70 (3)	−0·069 (*m*-)
5-Me	280 ± 20 (3)	0·78 ± 0·05 (3)	28 000 ± 570 (3)	720 ± 35 (3)	−0·170 (*p*-)
6-Me	940 ± 70 (4)	1·53 ± 0·11 (4)	45 000 ± 2 700 (3)	1 270 ± 100(3)	−0·069 (*m*-)
7-Me	150 ± 5 (3)	1·32 ± 0·09 (3)	20 000 ± 670 (3)	610 ± 75 (3)	−0·054 (*o*-)
4-Cl	1 170 ± 45 (5)	0·78 ± 0·02 (5)	309 000 ± 10 000 (3)	860 ± 10 (3)	+0·373 (*m*-)
5-Cl	1 920 ± 50 (5)	0·55 ± 0·06 (5)	221 000 ± 6 500 (3)	400 ± 3 (3)	+0·227 (*p*-)
6-Cl	4 750 ± 85 (5)	0·57 ± 0·08 (5)	397 000 ± 2 700 (3)	970 ± 45 (3)	+0·373 (*m*-)
7-Cl	370 ± 70 (3)	0·25 ± 0·05 (3)	16 700 ± 700 (3)	270 ± 10 (3)	+0·366 (*o*-)

ortho; if, alternatively, the main effect is exerted at C-3, then 5- and 7-substituents are *meta*, 6-substituents *para*, and 4-substituents *ortho*. The logarithms of the affinity constants, K_a, have been plotted against σ with both systems; for the latter system the correlation is very poor; for the former it is better, although still not good. Clearly, the effect of *Bz*-substitution on K_a is mainly exerted at the nitrogen atom of the indole nucleus.

Fig. 1 shows the logarithms of K_a, for degradation by both the cell-free enzyme and washed cell-suspensions, plotted against σ; the lines have been drawn by the least-squares procedure, excluding the points for the 7-substituents. The general conclusion can be drawn that the formation of the enzyme–substrate complex is facilitated by the presence in the benzene ring of electron-attracting chlorine atoms and hindered by the presence of electron-repelling methyl groups. The marked deviations, in the sense of much diminished enzyme–substrate affinity, observed with the 7-substituents is clearly a steric effect, hindering approach of an enzyme side chain to the indole nitrogen atom, and lends support to the view that this atom is involved in the enzyme–substrate combination. The necessity for the presence of an unsubstituted indole nitrogen for degradation by tryptophanase has been shown by Gooder & Happold (1953); these workers also showed free α-amino and α-carboxyl groups to be necessary; these findings are fully in accordance with the generalization (Rydon, 1948*b*) that an enzyme exhibiting the type of stereospecificity observed with tryptophanase must be attached to its substrate at, at least, three points.

Electron-attracting and electron-repelling *Bz*-substituents will, of course, have opposite effects on the hydrogen-bonding power of the imino group. Electron-attracting groups will favour hydrogen-bonding, through the hydrogen atom, to a suitable atom in an enzyme side chain carrying a lone pair of electrons; this is shown for 5-chlorotryptophan in (I). Electron-repelling groups, on the other hand, will facilitate hydrogen-bonding through the lone-pair electrons of the imino nitrogen to a suitable hydrogen atom in an enzyme side chain; this is illustrated for 5-methyltryptophan in (II). Our finding that electron-attracting substituents facilitate, and electron-repelling substituents hinder, formation of the enzyme–substrate complex indicates that the indole nitrogen is hydrogen-bonded to an enzyme side chain through its hydrogen atom, as in (I); this conclusion is in accordance with the mechanism of tryptophanase action put forward by Baker (1956).

Plots of log k_3', for both the cell-free enzyme and intact cells, against σ, no matter how chosen, show no satisfactory correlation and are accordingly not

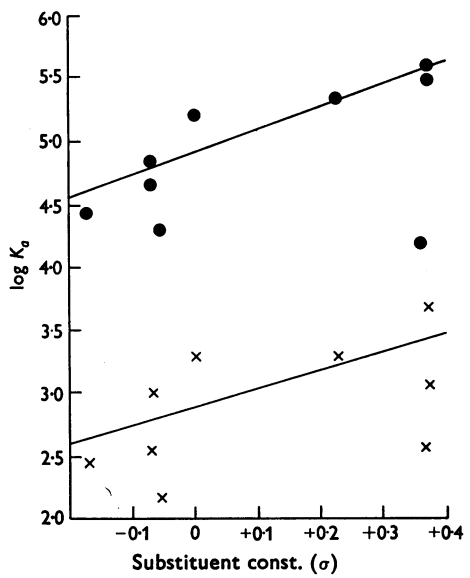
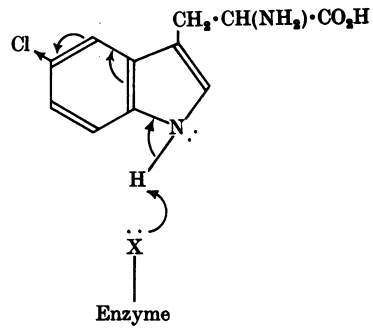
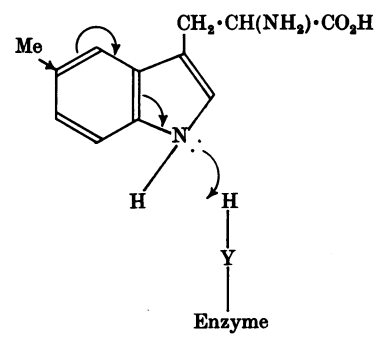


Fig. 1. Correlation of affinity constants, K_a, for the combination of *E. coli* tryptophanase with *Bz*-substituted tryptophans with the structures of the latter, as represented by the Hammett constants, σ, for the substituents. ×, Cell-free tryptophanase; ●, washed *E. coli* cells.



(I)



(II)

reproduced. It is, however, clear that k_3' has the highest value for unsubstituted tryptophan, both *Bz*-methyl and *Bz*-chloro substituents reducing the rate of breakdown of the enzyme–substrate complex, which is hindered by the presence in the benzene ring of both electron-repelling and electron-attracting substituents. It appears, therefore, that the rate of breakdown of the enzyme–substrate complex is determined by two oppositely directed processes, one opposed by electron-repelling substituents and one by electron-attracting substituents. These may tentatively be identified with the first and second stages of the breakdown of the tryptophanase–pyridoxal phosphate–tryptophan complex (Baker, 1956).

The K_a values for every one of the nine tryptophans studied are much higher for the intact cells than for the isolated enzyme; reference to Fig. 1 shows that, on average, K_a for the intact cell is about 100 times that for the isolated enzyme. This result is unexpected. Green (1951*a*) demonstrated that the oxidizing and phosphorylating activities of mitochondria are characterized by efficient co-ordination and by rates higher than would be expected on the basis of simple diffusion and random enzyme–substrate collision, and Peters (1955) found large differences between the fluorocitrate sensitivity of aconitase in kidney-particle fractions and in purified, cell-free preparations. Gale (1946) showed that the decarboxylases for arginine, ornithine and glutamic acid exhibit a somewhat greater affinity for their substrates in the intact cell than they do in cell-free preparations; he interprets this as indicating that the enzyme is damaged during the extraction process, but we have no evidence for such damage during the extraction of tryptophanase from *E. coli* cells.

There are two attractive explanations for the very large difference in K_a. First, possibly the tryptophanase system may be orientated in the intact cell in some particularly favourable manner. This view would accord well with Baker's (1953, 1956) conclusion that, in the formation of the enzyme–substrate complex, apotryptophanase presents a planar surface to the reactants; this is more likely to be realized within the living cell than in simple solution. Also, according to Green (1951*b*), there is evidence that some coenzymes are firmly attached to protein within the intact cell; this factor, too, might be expected to make the formation of the enzyme–substrate complex easier within the cell than in solution.

Secondly, it is possible that the concentration of substrate within the cell is greater than that in the surrounding medium; our results would require a 100-fold concentration. Our present knowledge of the concentration of amino acids by micro-organisms originates from the work of Gale (1947), who observed the concentration of a number of amino acids by Gram-positive bacteria. Taylor (1947) suggested that amino acids were not concentrated by Gram-negative bacteria, but it has subsequently been shown that L-valine, L-methionine and L-phenylalanine (Cohen & Rickenberg, 1955, 1956), and L-proline (Britten, Roberts & French, 1955), are, in fact, considerably concentrated by cells of *E. coli*, being in equilibrium with amino acids in the external medium; a supply of energy is, of course, required for such processes. The available evidence (Mitchell, 1957; Cohen & Monod, 1957) suggests strongly that the stereospecific sites responsible for the concentration of amino acids in *E. coli* are catalytic (translocases or permeases), rather than stoicheiometric. In view of the close chemical similarity of tryptophan and phenylalanine it seems that tryptophan, and substituted tryptophans, may also be capable of being concentrated within *E. coli* cells, especially as L-tryptophan can serve as an energy source for this organism.

SUMMARY

1. The degradation of tryptophan and the *Bz*-methyl- and -chloro-tryptophans by tryptophanase and by washed cell suspensions of *Escherichia coli* has been studied; the equilibrium constants, K_a, for the formation of the enzyme–substrate complexes and the first-order velocity constants, k_3, for their breakdown have been evaluated.

2. A fairly satisfactory correlation of K_a with the electronic properties of the substituents, as measured by the Hammett substituent constants, σ, has been obtained; the formation of the enzyme–substrate complex is facilitated by electron-attracting substituents in the benzene ring of the tryptophan molecule.

3. The velocity constants k_3' do not show satisfactory correlation with σ; however, both electron-attracting and electron-repelling substituents diminish k_3'.

4. The results are discussed in the light of current views on the mechanism of the degradation of tryptophan by tryptophanase.

5. The affinity constants K_a are about 100 times as great for washed cells of *E. coli* as for cell-free tryptophanase; possible explanations for this unexpected result are discussed.

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Physiological Studies on Acid Metabolism

7. MALIC ENZYME FROM *KALANCHOË CRENATA*: EFFECTS OF CARBON DIOXIDE CONCENTRATION*

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Green parts of certain succulent plants, including many members of the Crassulaceae, show a striking diurnal fluctuation in their organic acid content. This is largely a result of an accumulation of malic acid by night and its disappearance by day (Bennet-Clark, 1933). Thomas (1947) proposed that carbon dioxide is a metabolite in acid synthesis in addition to being, as many earlier investigators believed, a product of acid breakdown. This view was substantiated by extensive physiological experiments (Thomas, 1947, 1949; Thomas & Beevers, 1949; Thomas & Ranson, 1954) and by the demonstration that $^{14}CO_2$ was incorporated into a number of acids, but principally into malate, by crassulacean plants in the dark (Thurlow & Bonner, 1948; Varner & Burrell, 1950; Thomas & Ranson, 1954).

A known reaction (1) in which carbon dioxide is fixed and malate is synthesized is that catalysed by the malic enzyme (Ochoa, Mehler & Kornberg, 1947):

$$CH_3\cdot CO\cdot CO_2H + CO_2 + TPNH_2 \overset{Mn^{2+}}{\rightleftharpoons} CO_2H\cdot CH_2\cdot CHOH\cdot CO_2H + TPN \quad (1)$$

($TPNH_2$, reduced triphosphopyridine nucleotide, TPN, triphosphopyridine nucleotide.) This enzyme is of considerable interest in relation to crassulacean acid metabolism, for it is present in at least

* Part 6: Walker & Ranson (1958).