The Tryptophanase-Tryptophan Reaction. The Nature of the Enzyme-Coenzyme-Substrate Complex

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Baker & Happold (1940) first suggested a $C_{(\alpha)}$–$C_{(\beta)}$ fission in the tryptophan molecule as the essential process catalysed by tryptophanase. They showed that a free carboxyl group and an unsubstituted α-amino group were essential in the substrate, and since β-3-indolylglycine was not acted upon by tryptophanase they felt that the mechanism might involve oxidation at the β-carbon atom of the alanine side chain. Baker, Happold & Walker (1946) showed that an unsubstituted imino group was necessary in the indole nucleus since β-1-methyl-3-indolylalanine was not a substrate. These authors believed that the 3-carbon fragment which was separated from the indole nucleus was alanine, but Wood, Gunsalus & Umbreit (1947) showed that the reaction could be represented as

$$\text{tryptophan} \xrightarrow{\text{tryptophanase}} \text{indole} + \text{pyruvic acid} + \text{ammonia}$$

and that pyridoxal phosphate was the coenzyme for this system. These results were confirmed by Dawes & Happold (1949). The present work extends the previous findings of Baker *et al.* (1940, 1946) by determining the effect of the enzyme on an additional number of structurally related compounds and also the inhibitory effect of these compounds on the tryptophan-tryptophanase reaction. We feel that it is now possible to postulate that the system is a ternary complex of enzyme, coenzyme and substrate, and to suggest the most likely points of attachment between the three components.

MATERIALS AND METHODS

Cell-free extracts of the enzyme were obtained from cells of *Escherichia coli* by the method of Dawes & Happold (1949). Indole formed in the enzyme activity tests was determined by the method of Happold & Hoyle (1934). The rosindole colour formed by the addition of Ehrlich's reagent was measured in a Unicam D.G. Spectrophotometer at a wavelength of 5600 Å and is stable over a period of 15 min. Duplicate standard solutions can be estimated to within 1 μg. under the conditions used in the experiments.

Pyruvate was determined by the method of Friedemann & Haugen (1943), using the toluene-extraction procedure. The colours formed were measured in the spectrophotometer at a wavelength of 5200 Å. The standard colours for comparison were obtained from lithium pyruvate prepared by the method of Wendl (1931). Ammonia was determined by the microdiffusion method of Conway & O'Malley (1942).

Initial rate of enzyme action. This was determined by mixing 3 ml. of 0·0133 M phosphate buffer, pH 7·8, containing 10 μg. pyridoxal phosphate, and 1 ml. of enzyme solution and incubating for 10 min. at 37°. The substrate solution (1 ml. of 0·0196 M L-tryptophan which had been incubated separately) was then added over a period of 5 sec. and the reaction allowed to proceed for the required length of time; this was usually 5 min. but in the early experiments periods of up to 30 min. were employed. The reaction was stopped by the addition of 5 ml. of 10% (w/v) trichloroacetic acid (TCA) and the precipitated protein removed by centrifuging or filtration. The indole, pyruvate and ammonia were then determined on samples of the supernatant. The figure given is the average of two determinations. The initial rate was calculated as μg. indole formed/ml. TCA sample/min., measured over the first 5 min. of the reaction time.

EXPERIMENTAL AND RESULTS

Rate of indole production

The rate of enzymic production of indole from tryptophan is shown in Fig. 1, in which three different cell-free enzyme extracts from *Esch. coli* have been used; where indole production was approximately 20 μg. in 5 min., the rate of reaction

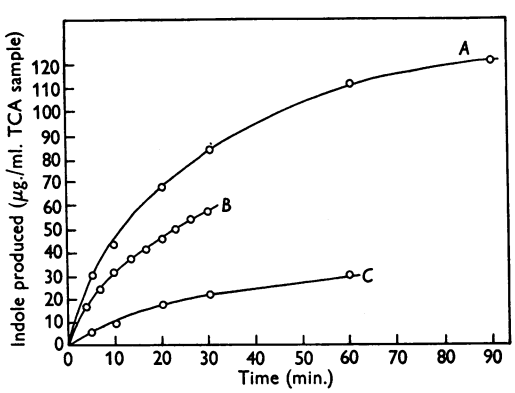

Fig. 1. Enzymic rate of indole production. *A*, *B* and *C* represent different enzyme preparations. Systems contained 1 ml. of enzyme, 1 ml. of L-tryptophan (0·0196 M) and 3 ml. of 0·0133 M Na_2HPO_4, KH_2PO buffer, pH 7·8.

was constant, so 5 min. was chosen as the time for the normal activity test. The rate of indole production after this point was not constant and the curve for enzyme solution *A* shows that 30 μg. indole were produced in the first 5 min., but only 9 μg. between 60 and 90 min. The decrease in rate in this experiment could not be accounted for solely in terms of decreasing substrate concentration.

Action of tryptophanase on derivatives of indole and indene

Activity tests were carried out by the above method using the following indole derivatives as substrates: 3-indolylacetic acid, β-3-indolylpropionic acid, β-3-indolylbutyric acid and β-3-indolylethylamine (all 2·12 mM), DL-α-amino-β-3-indolylbutyric acid (1·88 mM) and D-tryptophan (19·6 mM). In no case was any indole produced by the enzyme in 30 min. The inert nature of D-tryptophan had been indirectly shown by Woods (1935) for washed suspensions, by estimating the total indole produced from equimolar solutions of L- and DL-tryptophan. With the cell-free enzyme the results in Table 1 show that the rate of breakdown of solutions of L- and DL-tryptophan equimolar with respect to the L-form is the same and indicate that no inhibition occurs through the D isomer. This is studied in more detail later.

α-Amino-β-3-indenylpropionic acid (Groves & Swann, 1951) would be expected to behave similarly to tryptophan, provided the ring nitrogen atom was not essential to the reaction, producing indene, pyruvic acid and ammonia. L-Tryptophan and α-amino-β-3-indenylpropionic acid (1·96 mM) were compared as substrates in enzyme activity tests. The production of indole, ammonia and pyruvic acid from tryptophan was confirmed after 5 min. but no pyruvic acid or ammonia could be found from α-amino-β-3-indenylpropionic acid in reaction periods up to 30 hr. duration.

The inability of tryptophanase to cause any fission of the present group of compounds, and the earlier work of Baker & Happold (1940) and that of Baker *et al.* (1946) suggests strongly that the enzyme only acts on a compound containing an intact α-amino grouping, a carboxylic acid grouping and a ring nitrogen atom unsubstituted by alkyl groups. The failure to react with DL-amino-β-3-indolylbutyric acid stresses the importance of the 3-carbon side chain. The enzyme attacks only one stereoisomeric form of the substrate and requires the presence of the ring nitrogen atom for the reaction to occur. The nitrogen atom may be concerned in the combination of substrate and enzyme, in which case the indole nucleus may act as an inhibitor of the reaction. Wood *et al.* (1947) showed that added indole inhibited tryptophan breakdown, but Beerstecher & Edmonds (1951), using washed suspensions of *Esch. coli*, claimed that indole is probably required in catalytic amounts for the breakdown of tryptophan.

Table 1. *Action of tryptophanase on* L- *and* DL-*tryptophan*

	Final concentration of tryptophan (mM)					
	L	DL	L	DL	L	DL
	1·96	3·92	0·98	1·96	0·49	0·98
Time (min.)	Indole produced (μg./ml. TCA sample)					
5	17·5	18·0	14·0	13·6	8·0	7·0
10	33·4	33·6	—	—	—	—
15	40·5	42·8	—	—	—	—

Inhibition of tryptophanase by indole

We have found that the drop in the rate of indole formation by enzyme action after the first 5 min., which was not due solely to the drop in substrate concentration, was also not due to (*a*) loss of indole from the reaction vessels by volatilization or utilization, for periods up to 30 min., (*b*) enzyme denaturation during the reaction period since the activity of enzyme preparations incubated for periods up to 1 hr. before addition of substrate, did not drop, or (*c*), removal of the coenzyme from the reaction system since the initial rate did not vary with increases in the concentration of added coenzyme. Inhibition by indole seemed probable and was confirmed as follows. Indole and enzyme were incubated together for 10 min. before the addition of substrate. The concentration of L-tryptophan was 1·96 mM and that of the inhibitor, indole, 0·21 mM. The initial rate of indole production in the absence of added indole was 5·2 μg./min.; with the inhibitor present this dropped to 2·9 μg./min. The effect of varying the pre-incubation time of enzyme with indole upon the initial rate of the reactions was next tested for periods varying by 5 min. intervals from 5 to 30 min. There was no additional inhibitory effect with time after the first interval of time; equilibrium was quickly reached between the participants.

To study the type of inhibition involved, comparisons were made of the amount of inhibition when the substrate concentration was kept constant and the inhibitor concentration was varied, and vice versa. The results obtained with two different enzyme solutions are given in Table 2.

It should be noted that when indole is used as an inhibitor indole production is obtained by difference, the errors involved are increased and duplicate results vary by up to 2 μg., i.e. 0·4 μg. in the initial rate. With high substrate concentration the inhibitor concentration rapidly increases but in spite of this and the other experimental limitations the results show that at constant substrate concentra-

Table 2. *Effect of inhibitors on initial rate of reaction of tryptophanase with* L-*tryptophan*

Enzyme (1 ml.), 0·0133 M Na_2HPO_4, KH_2PO_4 buffer (2 ml.; pH 7·8) containing 10 μg. pyridoxal phosphate and inhibitor (1 ml.) were incubated together for 10 min. at 37°. L-Tryptophan (1 ml.) was then added and measurement of indole produced was made as in the Methods section. Concentrations of substrate and inhibitor are those obtaining after addition of substrate. The results were compared with those of controls containing water instead of inhibitor and the inhibition is expressed as the percentage decrease in the initial rate caused by the presence of inhibitor. Two different enzyme solutions (*a* and *b*) were used.

		L-Tryptophan in digest (mM)						
		7·0	1·96		0·98		0·49	
			a	*b*	*a*	*b*	*a*	*b*
Inhibitor	Inhibitor concn. (mM)	Inhibition (%)						
Indole	0·034	10	21	16	22	33	37	38
	0·068	32	57	33	73	51	87	94
3-Indolylacetic acid	0·068	—	0	0	—	—	—	—
	1·06	—	38	32	—	—	—	—
DL-α-Amino-β-3-indolylbutyric acid	1·88	33	—	—	—	—	66	—
β-3-Indolylpropionic acid	0·034	—	0	—	10	—	0	—
	0·068	—	0	—	5	—	0	—
	1·06	33	40	44	—	56	—	10
	2·12	54	—	59	—	73	—	91
β-3-Indolylethylamine	1·04	8	—	7	—	11	—	—
	2·07	11	—	10	—	12	—	—
D-Tryptophan	1·96	—	—	0	—	—	—	—
DL-β-1-Methyl-3-indolylalanine	3·49	—	—	0	—	—	—	—
	6·18	—	—	6	—	—	—	—

tion added indole causes inhibition. This increased inhibition is also produced at constant indole concentration by a decrease in substrate concentration. The results are consistent with competition by the inhibitor, indole, for the enzyme in place of the substrate. This presumably involves the ring nitrogen atom which is common to both structures, and in consequence other structural analogues of indole and tryptophan were investigated for the ability to inhibit tryptophan breakdown.

Structural analogues of L-*tryptophan and indole as inhibitors of tryptophan breakdown*

β-3-Indolylpropionic acid. This was tested in a similar manner to that previously described for indole. Inhibition of the reaction was found but there was no difference in the degree of inhibition when the order of mixing the participants was varied. The effects of varying the inhibitor and substrate concentrations as measured by the initial rate are also recorded in Table 2.

The results show that low concentrations of indolylpropionic acid have little effect; similar concentrations of indole produced substantial inhibition. Higher concentrations of the indole acid inhibited enzyme action. Results with other indolyl acids are also given.

β-3-Indolylethylamine. The indolylethylamine has only a slight inhibitory effect in concentrations at which the indolylpropionic acid inhibited strongly (Table 2).

D-*Tryptophan and β-1-methyl-3-indolylalanine.* As shown in Table 2, these do not inhibit tryptophanase.

Indene. This was prepared by distillation of a commercial sample (British Drug Houses Ltd.) under reduced pressure at 65°. A concentrated solution was prepared in ethanol and added to different enzyme preparations but only within a range in which the added ethanol caused no inhibition of the reaction. At 0·173 mM concentration, indene caused no decrease in the initial rate of indole formation with a substrate concentration of 0·196 mM. Indole in this concentration inhibited strongly.

α-Amino-β-3-indenylpropionic acid. This substance showed different effects as an inhibitor of the tryptophanase system according to the order in which the reactants were mixed. No, or little, inhibition was produced when the enzyme was added to a mixture of the substrate and test substance, but when the latter was pre-incubated with the enzyme, definite inhibition was found. This is shown by the results in Tables 3 and 4. α-Amino-β-3-indenylpropionic acid appears to inhibit the reaction when mixed with the enzyme and incubated for 30 min. prior to the addition of substrate. The amount of inhibition produced was small compared to that given by similar concentrations of indole acids.

Table 3. *Effect of α-amino-β-3-indenylpropionic acid on initial rate when incubated for 10 min. with substrate before addition of enzyme*

For experimental conditions see Table 2.

L-Tryptophan (mM)	α-Amino-β-3-indenylpropionic acid (mM)	Initial rate (μg. indole/ml. TCA sample/min.)
3·43	0	3·8
	2·45	3·4
1·96	0	4·0
	1·23	3·9
0·245	0	4·3
	0·49	4·2
	2·45	3·9

Table 4. *Effect of the incubation of α-amino-β-3-indenylpropionic acid with enzyme before the addition of substrate*

For experimental conditions see Table 2.

Time of pre-incubation (min.)	L-Tryptophan (mM)	
	1·96	0·49
	Initial rate (μg. indole/ml. TCA sample/min.)	
Enzyme		
5	5·1	3·3
30	5·0	3·5
Enzyme and α-amino-β-3-indenylpropionic acid ($1·23 \times 10^{-3}$ M)		
5	4·3	2·6
15	—	2·7
20	4·2	—
30	3·7	—

DISCUSSION

The previously postulated structural requirements necessary in a substrate for tryptophanase have been re-investigated using cell-free enzyme and the investigation has been extended by testing structural analogues of the substrate as inhibitors of enzyme action. In the previous and present studies, indole-3-substituted compounds were tested as substrates for the enzyme but no substance other than L-tryptophan has yet been found to yield indole by tryptophanase action. The results of the present inhibition experiments are summarized thus.

Inhibition was produced by indole, 3-indolylacetic acid, β-3-indolylpropionic acid and DL-α-amino-β-3-indolylbutyric acid.

No inhibition (or slight) was produced by indene, β-3-indolylethylamine, β-1-methyl-3-indolylalanine, D-tryptophan and α-amino-β-3-indenylpropionic acid.

The inhibition by indole was previously reported by Wood *et al.* (1947). This inhibition may account for the enzymic oxidation of tryptophan not proceeding to completion, the progressive increase in the concentration of indole halting the reaction. Of the final products of the reaction, namely, indole, pyruvic acid and ammonia, only indole inhibits, and indeed ammonium, potassium and rubidium ions increase the activity of the enzyme-coenzyme-substrate system (Happold & Struyvenberg, unpublished).

Fildes & Rydon (1947), in their studies on the tryptophan desmolase system of Tatum & Bonner (1944), suggested that a type of hydrogen bonding was formed between the ring nitrogen atom and two side chains of an enzyme molecule. Although the two enzyme systems are different, a similar suggestion might be applied to the system we have studied, although a double attachment between the ring nitrogen atom and two side chains of the enzyme molecule would not be involved.

The failure of the enzyme to react with β-1-methyl-3-indolylalanine would agree with the view that some sort of bonding occurs between the enzyme complex and the ring nitrogen atom and consideration of the structures of indole (whose inhibition of tryptophanase reaction is a function of indole and tryptophan concentrations) and indene (which does not inhibit) supports this assumption.

The failure of the enzyme to react with β-3-indolylethylamine and of this compound to inhibit indole formation by tryptophanase action supports the view that a carboxyl group is required for combination with the enzyme. β-3-Indolylpropionic acid, which possesses the carboxyl group of the usual substrate but not the α-amino group, is an inhibitor as might be expected if the acid group is involved in enzyme combination. The results show that the indole acid is not as good an inhibitor as indole itself, but since the acid possesses a side chain, a certain orientation on the surface of the enzyme would be necessary for inhibition which would not be required for inhibition by indole. Indene has no effect on the enzyme reaction but the addition of an alanine side chain to give α-amino-β-3-indenylpropionic acid, produces a compound which inhibits tryptophanase action, especially if it is pre-incubated with the enzyme before the addition of substrate. This may reflect a tendency for the side chain of the molecule to combine with the enzyme surface. The results with the two substituted propionic acids, taken together with the previous findings, suggest that combination in the tryptophanase system occurs between the apoenzyme and the carboxyl group of tryptophan. The apoenzyme would consequently be joined to the substrate by a two-point contact, by bonding with the ring nitrogen atom and through the carboxyl group in the side chain.

We are left with the position of the coenzyme pyridoxal 5-phosphate in the complex. The integrity of the α-amino group in tryptophan is essential to the complete tryptophanase system. If this group were attached directly to the apoenzyme we would expect β-3-indolylethylamine, which possesses an unsubstituted ring nitrogen atom and an α-amino group in the side chain, to inhibit the enzyme reaction strongly; in fact its inhibition is very weak. It seems reasonable to assume that β-3-indolylethylamine combines with the coenzyme, and since the tryptophanase reaction is inhibited by carbonyl reagents (Dawes & Happold, 1949) the logical assumption is that combination occurs between the α-amino group of the substrate and the aldehydic group of the pyridoxal phosphate. Combination between pyridoxal phosphate and amino acids during enzymic and non-enzymic reactions has often been suggested, and recently Baddiley (1952) showed that under certain conditions non-enzymic transamination could occur between α-amino acids, pyridoxal phosphate and a metal ion.

Umbreit & Wadell (1949) have shown that deoxypyridoxine, which antagonizes the action of pyridoxal phosphate and pyridoxine with *Streptococcus faecalis R*, has no effect upon the following free enzyme systems: tryptophanase, aspartic-glutamic transaminase and tyrosine decarboxylase. Deoxypyridoxine phosphate, however, inhibited the tyrosine decarboxylase system completely if added to the apoenzyme before the addition of coenzyme. This was taken as evidence that the method of combination between apoenzyme and coenzyme was by means of the phosphate group, possibly by hydrogen bonding, and Beechey & Happold (unpublished results) have now been able to show a similar inhibition when deoxypyridoxine phosphate is added to the apoenzyme of tryptophanase before the addition of pyridoxal 5-phosphate.

Tryptophan and pyridoxal phosphate give rise with tryptophanase to substrate-enzyme and coenzyme-enzyme saturation curves respectively (Wood *et al.* 1947; Dawes & Happold, 1949), and it can be assumed that each forms an intermediate complex with the enzyme resulting probably in a ternary complex. The logical interpretation of the present data suggests that the apoenzyme is bound to the coenzyme through the phosphate group of the latter while the coenzyme is associated with the substrate through the carboxyl group of the pyridoxal phosphate and the amino group of tryptophan, the latter being combined with the apoenzyme through the ring nitrogen atom and the terminal carboxylic acid group.

Finally, Baker (1953) has developed a possible model for the tryptophan-tryptophanase system which is based upon our experimental findings and of our acceptance of α-aminoacrylic acid as an intermediate in the degradation of tryptophan as suggested by Gale (1951). He has constructed molecular models of L- and D-tryptophan and pyridoxal 5-phosphate which show that (*a*) pyridoxal phosphate and tryptophan molecules fit neatly together to form a molecular complex in which the interacting aldehyde group of the former and the amino group of the latter are brought into close juxtaposition, and (*b*) in this complex with L-tryptophan (but *not* with the D-form), the various groups through which we suggest bonding with the apoenzyme occurs, all lie in one plane.

Thus geometry also indicates that, granted the previously mentioned groups are involved in the activity of the substrate-apoenzyme-coenzyme complex, the most probable associations are those we have suggested.

SUMMARY

1. A study of the effect of certain structural analogues of tryptophan on the tryptophan-tryptophanase reaction shows that indole and some indolyl acids inhibited the reaction strongly, while slight inhibition was given by α-amino-β-3-indenylpropionic acid when pre-incubated with the enzyme. Indene, D-tryptophan, β-3-indolylethylamine and β-1-methyl-3-indolylalanine either failed to inhibit or inhibited the reaction but slightly.

2. The enzyme system did not produce indole with any indole-containing substrate other than L-tryptophan, nor was ammonia or pyruvic acid produced from α-amino-β-3-indenylpropionic acid.

3. The relationship of these results to possible mechanisms of the enzyme reaction is discussed. It is suggested that the apoenzyme and coenzyme are linked through the phosphate group of the latter, whilst coenzyme and substrate are joined through the aldehyde group of the coenzyme and the amino group of the substrate. The substrate appears to be combined with the apoenzyme through the ring nitrogen atom and the terminal carboxyl group.

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REFERENCES

Baddiley, J. (1952). *Nature, Lond.*, **170**, 711.
Baker, J. W. (1953). *Biochem. J.* **55**, xxxii.
Baker, J. W. & Happold, F. C. (1940). *Biochem. J.* **34**, 657.
Baker, J. W., Happold, F. C. & Walker, N. (1946). *Biochem. J.* **40**, 420.

Beerstecher, E. & Edmonds, E. J. (1951). *J. biol. Chem.* **192**, 497.
Conway, E. J. & O'Malley, E. (1942). *Biochem. J.* **36**, 655.
Dawes, E. A. & Happold, F. C. (1949). *Biochem. J.* **44**, 349.
Fildes, P. & Rydon, H. N. (1947). *Brit. J. exp. Path.* **28**, 211.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Gale, E. F. (1951). *Bacterial Physiology*, p. 445. New York: Academic Press, Inc.
Groves, L. H. & Swann, G. A. (1951). *J. chem. Soc.* p. 1142.
Happold, F. C. & Hoyle, L. (1934). *Biochem. J.* **28**, 1171.
Tatum, E. L. & Bonner, D. (1944). *Proc. nat. Acad. Sci., Wash.*, **30**, 30.
Umbreit, W. W. & Wadell, J. G. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 293.
Wendl, W. B. (1931). *J. biol. Chem.* **94**, 717.
Wood, W. A., Gunsalus, I. C. & Umbreit, W. W. (1947). *J. biol. Chem.* **170**, 313.
Woods, D. D. (1935). *Biochem. J.* **29**, 640.

A Note on the Action of Fluoroacetate

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Liébecq & Peters (1949) showed that the inhibitory action of fluoroacetate on the oxidation of pyruvate and fumarate could not be accounted for by the postulated competitive inhibition of acetic acid oxidation (Bartlett & Barron, 1947). They demonstrated that citrate accumulated during the oxidation of fumarate and pyruvate and suggested that fluoroacetate after activation underwent a condensation to form a fluorotricarboxylic acid. This hypothesis has since been confirmed (Buffa, Peters & Wakelin, 1951) and the inhibitor, presumably fluorocitric acid, has been isolated in crystalline form (Peters, Wakelin & Buffa, 1951). These authors state that this is apparently not a simple case of competitive inhibition, because of the straight-line relation which was found between the active substance and the effect upon citrate disappearance. They used however only one concentration of citrate. Although the theory of competitive inhibition can only be applied with reserve to a multienzyme system contained in particles such as mitochondria, it seemed of interest to apply the method of Lineweaver & Burk (1934) to the system; we have therefore examined the effect of fluorocitrate on citrate oxidation by rabbit-kidney mitochondria at various concentrations of substrate and inhibitor. These experiments were done in 1950 during another investigation (Judah & Rees, 1953) and are published here in the hope that the results might be of interest to others.

Methods

Mitochondria from rabbit kidney were obtained by the method of Schneider (1948) using 0·25 M sucrose as the medium.

Oxygen uptake was measured manometrically in a Warburg apparatus in air at 38°. Initial velocities were taken as μl. O_2 uptake in the first 5 min. after 10 min. equilibration.

Citrate was estimated by the method of Weil-Malherbe & Bone (1949).

Materials

Adenosine 5′-phosphate obtained commercially was crystallized twice from hot water. By spectrophotometric analysis (Kalckar, 1947) and paper chromatography (Hanes & Isherwood, 1949) it was pure.

Sodium citrate was a commercial preparation.

Sodium fluoroacetate was provided by Dr R. A. E. Galley of the Agricultural Research Council.

Cytochrome *c* was prepared by the method of Keilin & Hartree (1937) and dialysed against distilled water.

Composition of the medium: inorganic phosphate 0·025 M, pH 7·4; adenosine 5′-phosphate 0·001 M; $MgSO_4$ 0·0067 M; KCl 0·025 M; cytochrome *c* 1×10^{-5} M; mitochondrial suspension in 0·25 M sucrose; and water to a final vol. of 3 ml.

RESULTS

During investigations on the action of fluoroacetate on citrate oxidation by rabbit-kidney mitochondria we observed that the inhibition was slight in the early part of the experiment and then progressed. Table 1 shows a typical experiment representative

Table 1. *Progressive inhibition of citrate oxidation by fluoroacetate*

System: standard medium as described in text. 10^{-3} M fluoroacetate; citrate, 30 μmoles. Enzyme, rabbit-kidney mitochondria. All present initially.

	O_2 uptake (μl.)	
	Fluoroacetate	No fluoroacetate
1st 10 min.	236	236
2nd 10 min.	95	203
3rd 10 min.	90	210