Activation of δ -Aminolaevulate Synthetase in Extracts of *Rhodopseudomonas spheroides*

By J. MARRIOTT,* A. NEUBERGER AND G. H. TAIT

Department of Chemical Pathology, St. Mary's Hospital Medical School, London W.2, U.K.

(Received 9 January 1970)

1. The spontaneous activation of δ -aminolaevulate synthetase in extracts from *Rhodopseudomonas spheroides* grown semi-anaerobically in the light requires oxygen and does not take place anaerobically in the dark. 2. Activation is completely prevented by azide or cyanide and is partially inhibited by chlorpromazine. 3. NADH delays activation, but when it has been oxidized by the extract activation begins at the normal rate and complete activation occurs. By contrast both the rate and the extent of activation are markedly decreased by even small amounts of carboxylic acids. 4. The inhibitory effects of succinate and citrate on activation can be prevented by malonate and fluorocitrate respectively. 5. These results suggest that for activation to occur some endogenous compound has to be oxidized via the electron transport chain. 6. Activation occurs under anaerobic conditions in the light, probably by photo-oxidation.

When suspensions of *Rhodopseudomonas spheroides* grown semi-anaerobically in the light are disrupted, the activity of ALA $\dagger$ synthetase is low initially. But if the homogenate is stored at 4°C a four- to five-fold increase in activity occurs within 1 h (Marriott, Neuberger & Tait, 1969). This activation is inhibited by citrate, α -oxoglutarate, succinate and β -hydroxybutyrate. No activation of ALA synthetase occurs in extracts of air-grown *R. spheroides*, or in extracts of cells that have been grown semi-anaerobically in the light and then oxygenated before harvesting. However the ALA synthetase activity of these extracts can be increased by addition of a dialysable component obtained from semi-anaerobically grown cells (Marriott, 1968).

In this paper factors affecting the activation of ALA synthetase in extracts of semi-anaerobically grown cells have been investigated.

MATERIALS AND METHODS

Chemicals. CoA, ATP and NADH were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K., monofluoroacetate from BDH (Chemicals) Ltd., Poole, Dorset, U.K., and chlorpromazine hydrochloride from May and Baker Ltd., Dagenham, Essex, U.K.

Methods. Cells of *R. spheroides* were grown and harvested, cell-free extracts were prepared, and ALA synthetase

activity was measured as described by Marriott *et al.* (1969). Potassium phosphate buffer (0.05M), pH 7.5, was used for suspending cells and for diluting extracts. NADH oxidation by cell-free extracts was followed by diluting portions of the incubation mixture at intervals and measuring the extinction at 340 nm.

Experiments under anaerobic conditions were performed in the following manner. Cell suspensions, buffers and reagent solutions were saturated with N₂ and cell suspensions were sonicated under a stream of N₂. The resulting homogenates were rapidly added to buffer under N₂ in stoppered tubes. Samples were taken at intervals for assay of ALA synthetase activity.

The oxygen electrode was purchased from Rank Brothers, Bottisham, Cambs., U.K.; oxygen uptake by whole cells and by homogenates was measured with the oxygen electrode connected to a Honeywell-Brown recorder (Motherwell, Scotland, U.K.; 1 mV full-scale deflexion). Succinoxidase activity was measured by following the uptake of oxygen in an incubation mixture containing 2.0 ml of 0.05M-phosphate buffer, pH 7.5, saturated with air at 24°C, extracts containing 1–3 mg of protein, and 0.1 ml of 1M-sodium succinate.

RESULTS

Requirement of oxygen for spontaneous activation of ALA synthetase. When semi-anaerobically grown cells are disrupted under anaerobic conditions and oxygen is excluded from the homogenate, no spontaneous activation occurs (Fig. 1). When oxygen is introduced, activation then occurs at the normal rate. Thus it appears that oxygen is required for activation.

* Present address: Tropical Products Institute, Gray's Inn Road, London W.C.1, U.K.

$\dagger$ Abbreviation: ALA, δ -aminolaevulate.

Fig. 1. Requirement for air in the spontaneous activation of ALA synthetase. A suspension of *R. spheroides* (100mg dry wt./ml) was sonicated and the homogenate, diluted fivefold, was stored at 4°C. After various times portions were assayed for ALA synthetase activity (O). Another suspension of *R. spheroides* was sonicated under N₂ and the diluted homogenate was stored at 4°C in tubes open to the air. At various times portions were assayed for ALA synthetase activity (Δ). A third suspension of *R. spheroides* was sonicated under N₂ and the diluted homogenate was stored at 4°C in stoppered tubes filled with N₂. After 60min, as indicated by the arrow, the stopper was removed and samples were assayed at intervals for ALA synthetase activity (□).

Table 1. Effect of inhibitors of electron transport on the activation of ALA synthetase

A suspension of *R. spheroides* (100mg dry wt./ml) was disrupted by sonication. Immediately after disruption portions of the homogenate were diluted with 0.05M-potassium phosphate buffer, pH 7.5, and inhibitors were added to give the final concentrations shown. In Expt. 1 the homogenate was diluted tenfold (giving a suspension containing about 5mg of protein/ml) and in Expt. 2 fivefold. The diluted samples were left at 4°C for 1 h and then assayed for ALA synthetase. A portion of diluted homogenate was also assayed immediately.

Additions	Concn. (mM)	ALA synthetase activity (nmol of ALA/h per mg of protein)	
		Expt. 1	Expt. 2
None		248	312
Chlorpromazine	0.5	226	—
	1.0	192	—
	2.0	164	—
	4.0	102	—
NaN ₃	1.0	—	57
KCN	1.0	—	40
Initial activity		59	50

The effect of inhibitors of electron transport on activation. Sodium azide (1mM) and potassium cyanide (1mM) completely inhibit activation of ALA synthetase (Table 1). Chlorpromazine, which blocks electron transport at the level of cytochrome *c* in mitochondrial systems (Dawkins, Judah & Rees, 1959), inhibits activation to a large degree in the presence of a relatively low concentration of protein (Table 1, Expt. 1). At the concentrations used to inhibit activation these compounds have little or no effect on the activity of ALA synthetase when they are added after activation has occurred. The succinoxidase activity of homogenates of *R. spheroides* is almost completely inhibited by 1mM-azide or -cyanide. Chlorpromazine also inhibits the succinoxidase activity; the extent of inhibition with a given concentration of chlorpromazine decreases as the concentration of protein in the assay is increased.

Condition of disruption of cells. After sonication of cells for 30s the ALA synthetase activity is low when measured immediately (Table 2); only a slight activation occurs when the homogenate is stored, whereas a marked activation occurs if the supernatant, obtained by centrifuging the homogenate at 25000g for 10min, is stored. When the time of sonication is increased the initial activity rises until with 3min sonication the initial activity is almost as high as that obtained when the homogenate and supernatant are left for 30min before being assayed. The amount of protein remaining in the supernatant also increases with the time of sonication. When sonication for 30s and 3min is performed in the presence of 10mM-azide the initial activities are the same as in the sample sonicated for 30s without azide, and no activation occurs. These results show that during sonication for 3min almost complete activation occurs, whereas a sample sonicated for a shorter period is activated completely only after centrifuging at 25000g and storage at 4°C. The period of sonication normally used was 2 min.

Mechanism of action of inhibitors of activation. Homogenates of *R. spheroides* contain active NADH oxidase and succinoxidase systems. When homogenates are mixed with succinate, citrate, α-oxoglutarate or β-hydroxybutyrate, the partial pressure of oxygen in the mixture drops to zero within about 2min as measured by the oxygen electrode. In view of the finding that oxygen is required for the activation of ALA synthetase to take place, it was considered likely that these organic acids inhibit activation by removing the oxygen dissolved in the homogenate. To test this hypothesis compounds that specifically inhibit the oxidation of particular organic acids have been tested (Table 3). Malonate has no effect on the spontaneous activation of ALA synthetase, but

Table 2. *Effect of time of sonication on ALA synthetase activity*

Suspensions of *R. spheroides* (20mg dry wt./ml) were sonicated for the times shown. Portions of the homogenates were assayed for ALA synthetase activity immediately and again after standing at 4°C for 30 min. Immediately after sonication portions of the homogenates were centrifuged at 25000g for 10 min. The supernatants were removed and after standing at 4°C for 20 min they were assayed for ALA synthetase activity.

Time of sonication (min)	Addition before sonication	ALA synthetase (nmol of ALA/h per ml)			Protein in supernatant (mg/ml)
		Homogenate		Supernatant	
		Initial	Final		
0.5	None	422	687	3230	4.4
1.0	None	760	1360	3940	5.6
2.0	None	2495	4100	4680	9.4
3.0	None	3470	4740	4270	9.4
0.5	NaN ₃ (10mM)	530	463	565	5.1
3.0	NaN ₃ (10mM)	582	517	532	8.6

Table 3. *Effect of malonate and succinate on the activation of ALA synthetase*

A suspension of *R. spheroides* (10mg dry wt./ml) was sonicated. A portion of the homogenate was assayed for ALA synthetase immediately. The compounds shown were added to other portions, and after standing at 4°C for 30 min they were assayed.

Additions	Concn. (mm)	ALA synthetase (nmol of ALA/h per mg of protein)	
		Expt. I	Expt. II
None (assayed immediately)	—	66	35
None	—	312	154
Succinate	10	68	49
Malonate	80	332	—
Succinate and malonate	10 and 80	312	98
Citrate	1	—	35
Citrate and malonate	1 and 80	—	35

final activity obtained (Fig. 2). Oxidation of NADH is rapid even at 4°C and the onset of activation coincides with the completion of this reaction (Fig. 3). The progress of activation in the presence of limiting concentrations of carboxylic acids is completely different. The results reported in Fig. 4 for β -hydroxybutyrate are typical; they show that amounts of β -hydroxybutyrate sufficient to inhibit slightly the initial rate of activation also decreases the maximum activity reached.

Photo-activation of ALA synthetase. When homogenates of sonicated *R. spheroides*, in which activation of ALA synthetase has been prevented by additions of sodium azide, are illuminated, a gradual increase in the activity of ALA synthetase occurs (Table 5). Although this effect has not been investigated in detail it is probable that photo-oxidation occurs. Preliminary results show that activation brought about in this way is inhibited by addition of citrate.

DISCUSSION

when it is added along with succinate it antagonizes the inhibitory effect of succinate. Malonate does not interfere with the inhibition of activation caused by citrate. Since fumarate, the product of oxidation of succinate, has no effect on activation it seems likely that succinate acts by using up the oxygen in the system.

Sodium monofluoroacetate was added to cultures of cells growing semi-anaerobically in the light. After 1 h the cells were harvested. In extracts of these cells activation of ALA synthetase occurs in the normal manner (Table 4). This activation is inhibited by succinate but not by citrate.

Addition of NADH to homogenates causes a lag before activation begins, but it does not affect the

Previous work (Marriott, 1968; Marriott *et al.* 1969) has shown that the spontaneous activation of ALA synthetase in extracts of *R. spheroides* is controlled by at least two low-molecular-weight compounds, which have been called 'activator' and 'inhibitor' respectively. These compounds have not been identified nor have their modes of action been elucidated. The present results demonstrate that oxygen is required for activation to occur, and that succinate and other compounds previously shown to inhibit activation do so by competing for oxygen. Malonate and fluorocitrate (presumed to be formed *in vivo* from fluoroacetate), which specifically inhibit the oxidation of succinate and citrate respectively, block the inhibitory effects of these compounds on the activation of ALA synthetase

Table 4. *Effect of citrate on the activation of ALA synthetase in extracts of cells grown in the presence of monofluoroacetate*

Sodium monofluoroacetate to a concentration of 10mM was added to a culture of *R. spheroides* growing semi-anaerobically in the light. After 1 h the culture was harvested, and the cells were washed and suspended to 100mg dry wt./ml. The suspension was sonicated and then diluted fivefold. Portions of the resulting homogenate were assayed immediately and after standing at 4°C for 30min. Succinate and citrate were added to portions of the homogenate immediately after sonication, and after standing at 4°C for 30min they were assayed.

Additions	Concn. (mM)	ALA synthetase (nmol of ALA/h per mg of protein)			
		Expt. 1		Expt. 2	
		Normal cells	Fluoroacetate-grown cells	Normal cells	Fluoroacetate-grown cells
None (assayed immediately)	—	26	25	29	32
None	—	138	133	122	162
Citrate	1	40	93	40	148
Succinate	10	39	37	29	68

Fig. 2. Inhibition by NADH of the activation of ALA synthetase. A suspension of *R. spheroides* (100mg dry wt./ml) was sonicated and diluted fivefold. The homogenate was divided into four portions. To three of them were added NADH to concentrations of 0.5mM (Δ), 1mM ($\square$) and 2mM ($\bullet$). The fourth sample ($\circ$) had no addition made. The samples were stored at 4°C and at various times samples were assayed for ALA synthetase activity.

Fig. 3. Rates of oxidation of NADH and of activation of ALA synthetase. A suspension of *R. spheroides* (100mg dry wt./ml) was sonicated, diluted fivefold, and NADH was added (final concn. 2mM). The homogenate was stored at 4°C and at various times portions were assayed for the amount of NADH remaining ($\circ$) and for ALA synthetase activity ($\bullet$).

compound which is oxidized is the inhibitor and work to identify it is in progress.

(Tables 3 and 4). Fig. 3 shows that activation of ALA synthetase does not begin until all the added NADH has been oxidized. Inhibition of activation by anaerobiosis and by addition of azide, cyanide or chlorpromazine suggests that for activation to occur some compound has to be oxidized, the oxidation being coupled to the electron-transport system. In the absence of oxygen activation occurs in the presence of light, probably by photo-oxidation of this compound. It may be suggested that the

Competition for oxygen can also explain the results in Table 2. During sonication air is being taken up by the mixture; the longer the sonication the more air is taken up and therefore the more rapid is the activation. In addition, the longer the sonication the greater is the extent of cell disruption. It has been shown, with the oxygen electrode, that a given concentration of whole cells utilizes oxygen very much more rapidly than the same concentration of cells after disruption (G. H. Tait, unpublished work). This is presumably because in whole cells the oxidizable substrates are concentrated in the

Fig. 4. Inhibition of activation of ALA synthetase by DL- β -hydroxybutyrate. A suspension of *R. spheroides* (100mg dry wt./ml) was sonicated and diluted fivefold. The resulting homogenate was divided into four portions. To three of them were added DL- β -hydroxybutyrate to concentrations of 2mM (□), 4mM (Δ) and 8mM (●). The fourth sample had no addition (○). The samples were stored at 4°C and at various times portions were assayed for ALA synthetase activity.

cells, whereas on disruption they are diluted throughout the reaction mixture. Thus a sample of cells sonicated for only 30s still contains a significant proportion of whole cells, and activation of ALA synthetase released from broken cells can only occur if the whole cells are removed by centrifuging.

The differences in the patterns of activation of ALA synthetase after adding NADH (Fig. 3) or carboxylic acids (Fig. 4) cannot be readily explained. Although added NADH is completely oxidized and activation can occur normally, the

Table 5. *Effect of illumination of an extract of R. spheroides on ALA synthetase activity*

A suspension of *R. spheroides* (100mg dry wt./ml) was sonicated, the homogenate was diluted to give a protein concentration of 5mg/ml and NaN_3 was added to a concentration of 10mM. Part of the suspension was stored at 4°C in the dark and part was illuminated at 4°C. At various times portions were assayed for ALA synthetase activity.

Time at 4°C (h)	ALA synthetase (nmol of ALA/h per mg of protein)	
	Dark	Light
0	73	67
1	68	134
2	62	200

carboxylic acids may not be completely oxidized and may act as redox buffers and in some way prevent the proper conditions for activation from being attained.

The involvement of both oxygen and light in bringing about changes in the activity of ALA synthetase in cell-free extracts of *R. spheroides* seems to be of importance since this may be the way, or at least one of the ways, in which they exert their well-known controlling effects on the biosynthesis of bacteriochlorophyll in the whole cell (Cohen-Bazire, Sistrom & Stanier, 1957).

We are indebted to Mr S. Ladeji for skilled technical help.

REFERENCES

Cohen-Bazire, G., Sistrom, W. R. & Stanier, R. Y. (1957). *J. cell. comp. Physiol.* **49**, 25.
Dawkins, M. J. R., Judah, J. D. & Rees, K. R. (1959). *Biochem. J.* **72**, 204.
Marriott, J. (1968). *Biochem. Soc. Symp.* **28**, 61.
Marriott, J., Neuberger, A. & Tait, G. H. (1969). *Biochem. J.* **111**, 385.