MICROBIAL PRODUCTION OF AMINO ACIDS

IV. Studies on the Pathway of Alanine Biosynthesis in *Arthrobacter* Sp. $C_{19}d$

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ABSTRACT

The pathway of alanine biosynthesis by *Arthrobacter* sp. $C_{19}d$, which accumulates large quantities of DL-alanine in a molasses based medium, has been worked out using a metabolic inhibitor hydroxylamine hydrochloride, tracer techniques and cell-free extract procedures. The presence of alanine dehydrogenase has been demonstrated by way of its specificity of L-alanine as its substrate and NAD as its cofactor. The enzyme shows more than 80% inhibition to PCMB (para chloromercury benzoate). The evidences accrued indicate the reductive amination of pyruvate by alanine dehydrogenase as the main pathway of alanine biosynthesis in this organism.

ACCUMULATION of DL-alanine in large quantities by *Arthrobacter* sp. $C_{19}d$ in synthetic as well as in molasses based media[1-2] has been described earlier. In this communication the possible pathway of alanine biosynthesis in this organism is indicated. It has been postulated that biosynthesis of L-amino acids occurs by reductive amination of α-keto acids catalysed by amino acid dehydrogenases. However, for a long time, the only amino acid dehydrogenase shown to occur in nature is the glutamic acid dehydrogenase described by von Euler *et al.*[3]. Later, however, the occurrence of alanine dehydrogenase in *Bacillus subtilis* was demonstrated by Wiame and Pierard[4]. Subsequently not only its presence in a number of other micro-organisms[5-7] but also its dependence on NAD and specificity to L-alanine in all cases were demonstrated. However, its presence in animal and plant tissues is yet to be detected; therefore, its role in amino acid metabolism, from the point of view of comparative biochemistry, should await further elucidation.

MATERIALS AND METHODS

Medium.—The synthetic medium used in the various experiments had the following per cent composition: glucose 7·5; urea 1·0; K_2HPO_4 0·1; KH_2PO_4 0·05; $MgSO_4.7H_2O$ 0·025; $FeSO_4.7H_2O$ 0·001%; $MnSO_4.H_2O$ 0·001 and yeast extract 0·02. The culture was grown on a rotary shaker at 30° C, in 500 ml Erlenmeyer flasks containing 100 ml medium.

Isotope studies.—Labelled compounds, *viz.*, glucose-1-^{14}C, glucose-2-^{14}C, glucose-6-^{14}C, and pyruvate-U-^{14}C were obtained from Bhabha Atomic Research Centre, Trombay, Bombay. These were incorporated into the medium and after inoculation with *Arthrobacter* $C_{19}d$, the flasks were incubated on a rotary shaker and samples drawn at intervals of 12 hr and analysed for distribution of radioactivity in the amino acid fractions using chromatographic technique. The radioactive counts were taken in Beckman LS 100, liquid scintillation counter, using the solvent PPO-PoPoP in toluene.

Manometric studies.—Cells from a 48 hr old culture grown on a rotary shaker were harvested by centrifugation, washed twice with M/15 Phosphate buffer (pH 7·5) and suspended in the same buffer. The cell density was adjusted in such a way that each 1 ml aliquot contained approximately 100 mg of wet cells which were used immediately in manometric studies. The manometric experiment was carried out in a conventional way as described by Umbreit *et al.*[8]. The respiratory substrates employed were glucose, citrate, isocitrate, glyoxalate, succinate, α-keto-glutarate, malate, fumarate, pyruvate and acetate. The readings were recorded at intervals of 10 min.

Preparation of cell-free extract.—Cells grown for 48 hr in the synthetic medium mentioned above were washed free of medium with distilled water and the wet mass of cells were pressed between pads of filter-paper. Weighed quantities of these cells were suspended in 0·1 M Tris-HCl buffer (pH 7·5) in 1 : 10 proportion and subjected to sonic disruption in a 10 K oscillator for 30–40 min at maximum amperage. During sonication the temperature was kept below 5° C by circulating ice-cold water round the jacket. The sonicate was then centrifuged at 13,000 × g for 30 min in a Sorvall centrifuge at 0° C and the clear supernatant was dialysed overnight at 4–5° C against 0·05 M Tris-HCl buffer (pH 7·5) with 2–3 changes. The cell-free extract was then stored in deep freeze till required.

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Assay methods.—The methods used for the assay of various enzymes were those followed by Shiio *et al.*[9] and for alanine dehydrogenase assay that of Yoshida and Freese[10] with slight modifications, where necessary. The details of the reaction mixtures for each enzyme are given below. The reaction mixture contained the following reactants in micromoles, in a final volume of 3 ml.

Assay Mixtures for Enzymes

1. *Alanine dehydrogenase.*—For reductive amination of pyruvate, the reactants employed were sodium pyruvate 50; NADH 2; Tris-HCl buffer (pH 8·4) 100; $(NH_4)_2SO_4$ 30; alcohol dehydrogenase 0·5; ethyl alcohol 25; and cell-free extract 0·5 ml (1·9 mg protein). For oxidative deamination of alanine, it contained L-alanine 50; NAD 2; carbonate-bicarbonate buffer (pH 9·8) 100; and cell-free extract (1·9 mg protein).
2. *Glutamic acid dehydrogenase.*—Potassium α-ketoglutarate 20; sodium isocitrate 1·0; phosphate buffer (pH 7·6) 100; NADPH 1·0; $(NH_4)_2SO_4$ 30; $MnSO_4$ 0·5 and cell-free extract (1·9 mg protein).
3. *Transaminases.*—L-amino acids 25; potassium α-ketoglutarate or sodium pyruvate 25; Tris-HCl buffer (pH 8·4) 100; pyridoxal phosphate 0·2; and cell-free extract (1·9 mg protein).
4. *Isocitratase.*—Sodium isocitrate 25; $MgCl_2$ 5; Cysteine HCl 1·5; Tris-HCl buffer (pH 7·5) 100; and cell-free extract (1·9 mg protein).
5. *α-Ketoglutaric acid dehydrogenase.*—Potassium α-ketoglutarate 25; CoA 0·2; NADP 0·4; glutathione 9; phosphate buffer (pH 7·5) 100; and cell-free extract (1·9 mg protein).
6. *Malate synthetase.*—Na-glyoxalate 5; acetyl CoA 0·2; $MgCl_2$ 5; Tris-HCl buffer (pH 7·5) 100; and cell-free extract (1·9 mg protein).

In all cases the reaction mixtures were analysed for the products formed rather than with the usual spectrophotometric methods. The enzyme protein was determined by the method of Warburg and Christian[11].

After incubating the reaction mixtures in small test tubes at 37° C for 30 min, the reaction was stopped by keeping the tubes in boiling water for 5–10 min and the reaction mixture was analysed for the products formed. For the identification and estimation of amino acids, the supernatant was adjusted to pH 2·0 and passed through a column of amberlite IR-120 (H^+). After washing the column with distilled water, the adsorbed amino acids were eluted with 0·1 N ammonium hydroxide. The eluate was concentrated to a convenient volume and used for chromatography. The estimation of alanine and other amino acids was carried out by the method described by Giri *et al.*[12].

Pyruvate formed was identified as its 2–4-dinitrophenyl hydrozone by paper chromatography and its estimation was carried out according to the method of Friedman and Haugen[13].

The organic acids formed were obtained in their free state using amberlite IRA-400 (CO_3^{--} form) and Dowex 50 (H^+) columns and were chromatographed on Whatman No. 1 filter paper using the solvent, butanol : formic acid : water (4 : 1 : 5). Bromophenol blue (0·05% ; pH 7·0) was used to develop colour.

Results

1. *Effect of Sodium arsenite and Hydroxylamine hydrochloride on Alanine accumulation*

Arsenite is known to inhibit glucose metabolism at the stage where pyruvate enters the tricarboxylic acid cycle leading to pyruvate accumulation (Shiio, *et al.*[9]). Addition of arsenite to the growing culture of strain C_{19}d resulted in the accumulation of pyruvate (600 μg/ml) and arrested the growth of the organism completely (Fig. 1). Besides,

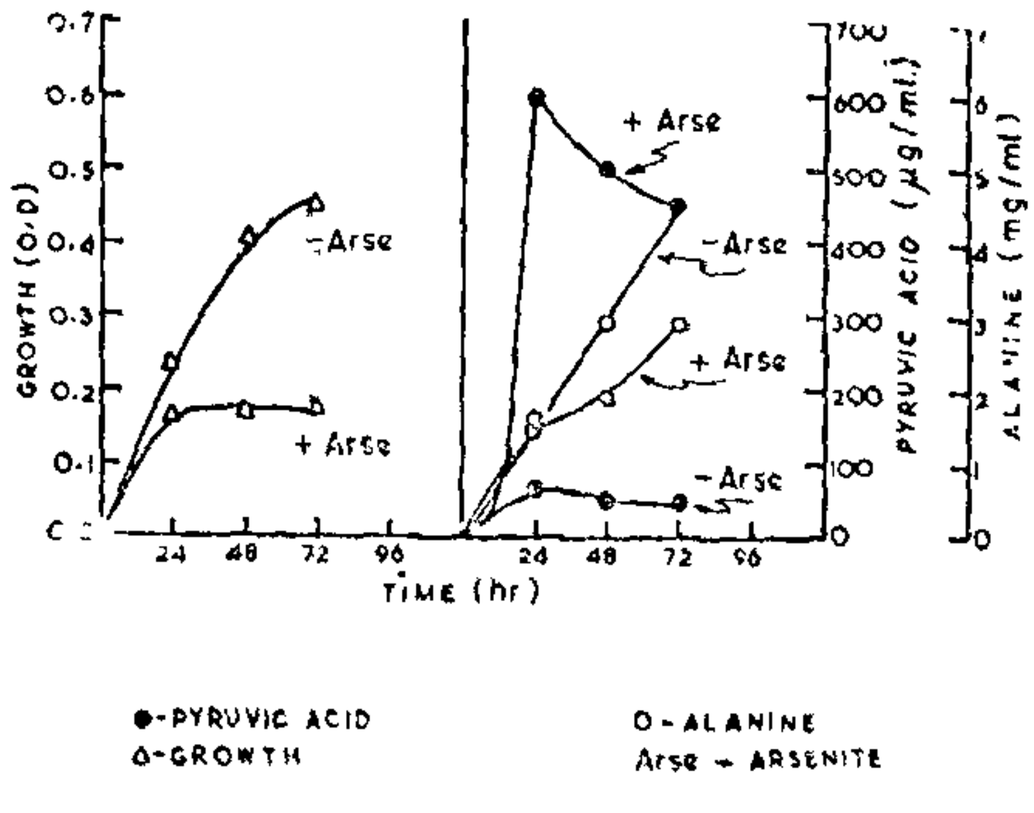

Fig. 1. Effect of sodium arsenite on glucose metabolism and alanine production by *Arthrobacter* sp. C_{19}d.

alanine synthesis was affected during the first 36 hr of arsenite incorporation, though later on as the pyruvate concentration started coming down, alanine formation increased appreciably without any increase in growth, indicating thereby the conversion of pyruvate to alanine. The slow conversion of pyruvate to alanine evidenced in the beginning was perhaps due to high concentrations

of pyruvate and the unfavourable pH 6·7 (optimum 7·5–8·2) caused by pyruvate accumulation. It is of interest to note here that pyruvate upto 65 μg/ml got accumulated even in control where no arsenite was added and offers a plausible explanation for alanine accumulation by this culture. The above result, though not a positive proof for reductive amination of pyruvate, is suggestive of that possibility.

To ascertain the possibility of transamination being the major route of alanine synthesis in this organism, hydroxylamine hydrochloride was used in the medium to inhibit this process in the growing culture. The results showed (Table I) that alanine yield was significantly high (9·2 mg/ml) wherein hydroxylamine hydrochloride was present at 10^{-4} M concentration as compared to control (7·0 mg/ml). In case alanine formation was the outcome of transamination of pyruvate with any of the amino acids, alanine yields should have been much lower than that of the control. It may be pointed out that the concentrations of hydroxylamine hydrochloride between 10^{-4} and 10^{-3}, in fact, reduced the growth of the organism as well as the yield of alanine and 10^{-2} M concentration proved actually inhibitory to the growth of the organism.

TABLE I

Inhibition of transamination by hydroxylamine hydrochloride in growing cultures

Hydroxylamine hydrochloride added (Molar)	Time of incubation (hr)	pH	Growth (O.D.)	Alanine formed (mg/ml)
0·0001 (10^{-4})	24	8·2	0·24	0·7
	48	8·3	0·52	5·0
	72	8·3	0·64	9·2
0·0005	24	8·2	0·32	2·5
	48	8·5	0·54	6·0
	72	8·5	0·55	2·6
No addition	24	8·2	0·28	0·6
	48	8·3	0·46	3·8
	72	8·3	0·55	7·0

Synthetic medium : glucose 7·5%; urea 0·75%; yeast extract, 0·02%; grown in 500 ml flasks containing 100 ml medium, on a rotary shaker at 30° C.

2. *Isotopic Studies*

The results shown in Table II indicate that glucose-1-^{14}C and glucose-2-^{14}C were not incorporated into the alanine fraction, whereas traces of the label were detected in the glutamic acid fraction. Even after 36 hr incubation, no radioactivity appeared in alanine fraction from glucose labelled at these two positions, whereas radioactivity from glucose-6-^{14}C and pyruvate-U-^{14}C appeared immediately in the alanine fraction though not in the glutamic acid formed. By 36 hr of incubation nearly 77% of the radioactivity of pyruvate incorporated in the medium appeared in the alanine fraction itself and only in small amounts appeared in the glutamic acid fraction.

These results again support the assumption of reductive amination of pyruvate rather than that of transamination route. If transamination were to be the main route of alanine synthesis, considerable amount of radioactivity would have appeared in the glutamic acid fraction within even a few hours of incubation, particularly where glucose-

TABLE II

Tracer studies using various isotopes

Labelled compounds	Total counts added	Radioactivity (c.p.m.)			
		24 hr		36 hr	
		Ccunts alanine fraction (per ml)	Counts in glutamic acid fraction (per ml)	Counts in alanine fraction (per ml)	Counts in glutamic acid fraction (per ml)
Glucose–1–^{14}C	1,95,000	0	750	0	1200
Glucose–2–^{14}C	1,73,000	0	700	0	1050
Glucose–6–^{14}C	1,95,000	900	0	1560	150
Pyruvate–U–^{14}C	1,40,800	990	0	2160	400

Culture grown in 250 ml flask with 50 ml synthetic medium

6-^{14}C was present in the medium. On the other hand, the result obtained almost rule out the possibility of transamination having any major role in alanine synthesis. The fact that glucose labelled at 1st and 2nd carbon was not incorporated into alanine suggests that the first half of the glucose molecule, unlike the second half, was metabolised in a route different from the usual EMP pathway.

3. *Cell-free Extract Studies*

In order to confirm the observations made in the above experiments, assay systems for various enzymes were set up using cell-free extract of the organism as the enzyme source.

(*a*) *Alanine dehydrogenase.*—A regenerating system for NADH was found to be essential due to the presence of NADH oxidase in the cell-free extract; when this was included in the assay mixture (No. 1) alanine formation was considerable. As may be observed from Table III more

TABLE III

Alanine dehydrogenase activity

Substrate given (μ moles)		Product formed (μ moles)		Substrate found (μ moles)	
Pyruvate	Alanine	Pyruvate	Alanine	Pyruvate	Alanine
50	..	..	27	19·1	..
..	50	0·8	..	..	49·0

than half the substrate given was converted to alanine within 30 min. The controls without pyruvate or NADH did not show any alanine formation. When NADPH, instead of NADH, was included in the assay mixture along with a regenerating system for it, there was no alanine formation. Addition of PCMB (*p*-chloromercury benzoate) to the assay mixture containing NADH (with a regenerating system), at 10^{-3} M concentration was found to inhibit the enzyme activity by over 80%.

The specificity of the enzyme for NADH indicates the presence of alanine dehydrogenase in this organism and tends to confirm the earlier observation that the reductive amination of pyruvate is the main route of alanine synthesis.

The oxidative deamination of L-alanine was also tried. However, the reaction rate was very slow and only a small amount of pyruvic acid was formed in the assay mixture (Table III).

(*b*) *Glutamic dehydrogenase.*—Test for the presence of this enzyme was made with a view to ascertain whether the enzyme by itself can catalyse the reductive amination of pyruvate to alanine, instead of alanine dehydrogenase doing so, since there are reports of such a possibility in some microorganisms (Tomkins *et al.*, 1961[14]). However, the activity of this enzyme was very low and only a small amount of glutamic acid was formed (2·3 moles) when 20 μm of α-ketoglutarate and adequate NADPH were present in the assay mixture (No. 2). When α-ketoglutarate and NADH were added there was no glutamic acid formation. This indicates that glutamic acid dehydrogenase is specific for NADPH and is different from the enzyme (alanine dehydrogenase) catalysing the reductive amination of pyruvate.

(*c*) *Transaminases.*—Transamination between pyruvate and L-amino acids, and α-ketoglutarate and alanine were then examined (No. 3). As shown in Table IV, transamination reaction between

TABLE IV

Transamination reactions

Transamination between	Substrate given (μ moles)	Product formed (μ moles)
Pvruvate-L-amino acids	25–25	Negligible (alanine)
α-Ketoglutarate-alanine	25–25	4·6 (glutamic acid)

pyruvate and 16 L-amino acids took place to a negligible extent only, whereas small amount of glutamic acid was formed when α-ketoglutarate and alanine were present in the assay mixture. The absence of transamination between pyruvate and L-amino acids, particularly glutamic acid, goes to show that the main route of alanine synthesis in this organism is not through transamination and that the low activity of alanine-α-ketoglutarate transaminase accounts for both the accumulation of alanine and the small amount of glutamic acid always detectable in the culture broth along with alanine.

(*d*) *α-Ketoglutaric acid dehydrogenase.*—The activity of this enzyme determines to a great extent the accumulation or otherwise of α-ketoglutarate in the cell. α-Ketoglutarate is known to be the precursor for glutamic acid formation. Since low activity of glutamic dehydrogenase and alanine-α-ketoglutarate transminase was clearly evidenced in the above experiments, it was of interest to see the extent to which supply of α-ketoglutarate to the above two reactions would provide for the formation of glutamate. Again, product formation of this reaction was looked into using chromatographic technique for identification of products. Within 10 min of the reaction (assay mixture No. 5)

appreciable amounts of succinic acid was found in the reaction mixture. Good activity of this enzyme, as evidenced by the above result, indicates that the chances of α-ketoglutarate supply for the above two reactions are rather limited.

(4) *Manometric experiments.*—Oxidation of tricarboxylic acid cycle intermediates by this organism was followed with a view to understand the role of glyoxalate bypath, if any, in the accumulation of pyruvic acid. The results showed very low oxidation values for isocitrate and malate, whereas oxidation values for glucose, fumarate, citrate, succinate and α-ketoglutarate were high (Figs. 2 *a* and 2 *b*). Pyruvate oxidation was rather low as compared to above substrates. Surprisingly, oxidation of acetate was good but glyoxylate remained unoxidised. Since the entry of glyoxalate and isocitrate into the cells are affected under high pH conditions (8·0–8·2 used in the experiment), the oxidation of these substrates were studied with cell-free extracts (assay methods) and the products formed were analysed chromatographically. In the case of isocitrate as the substrate (Assay mixture No. 4), appreciable amounts of glyoxalate and small amounts of malate were detected, whereas with glyoxalate as the substrate, (Assay mixture No. 6), appreciable amounts of malic acid and traces of oxalacetate were detected. The results not only point to the oxidation of glyoxalate and isocitrate by this organism, but also the participation of "glyoxylate bypath" along with usual tricarboxylic acid cycle. However, it is difficult to estimate from the above observations the extent of operation of these two cycles or the predominance of one cycle over the other on the role of each cycle in the accumulation of pyruvic acid. More detailed studies are needed before any definite conclusions can be drawn.

Discussion

The results of the experiments give evidence for the reductive amination of pyruvate to alanine through alanine dehydrogenase, particularly the cell-free extract studies which showed the specificity of this enzyme to NADH to catalyse the above reaction as well as the inhibition of its activity by PCMB. The fact that NADPH cannot activate alanine dehydrogenase shows that it is different from glutamic acid dehydrogenase which is specific for NADPH in the organism. The low activity of glutamic dehydrogenase in this organism and its specificity to NADPH go to show that it is different from the enzyme catalyzing the reductive amination of pyruvate to alanine as the latter enzyme is specific to NADH and has a very high activity. Hence the possibility of glutamic dehydrogenase acting as alanine dehydrogenase under suitable conditions, as proposed by Tomkins *et al.*[14], does not hold good for this organism and they appear to be different altogether since their rate of reaction, substrate specificity and co-factor requirements are distinctly different. The second possibility of a coupled action by α-ketoglutaric acid-alanine

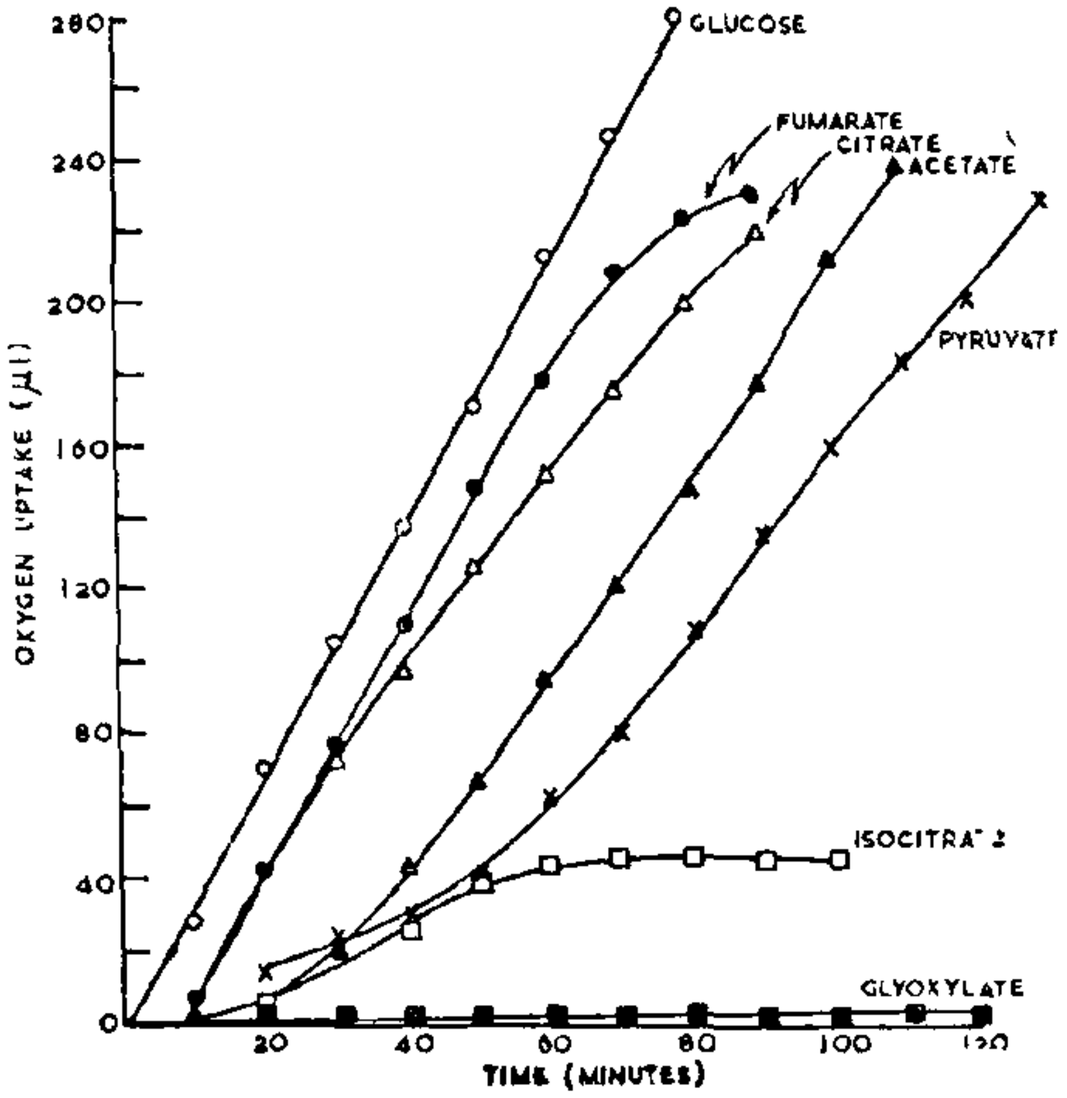

FIG. 2 *a*. Oxygen uptake from the oxidation of glucose, pyruvate and tricarboxylic acid cycle intermediates by washed cells of *Arthrobacter* strain C_{19}d grown on glucose.

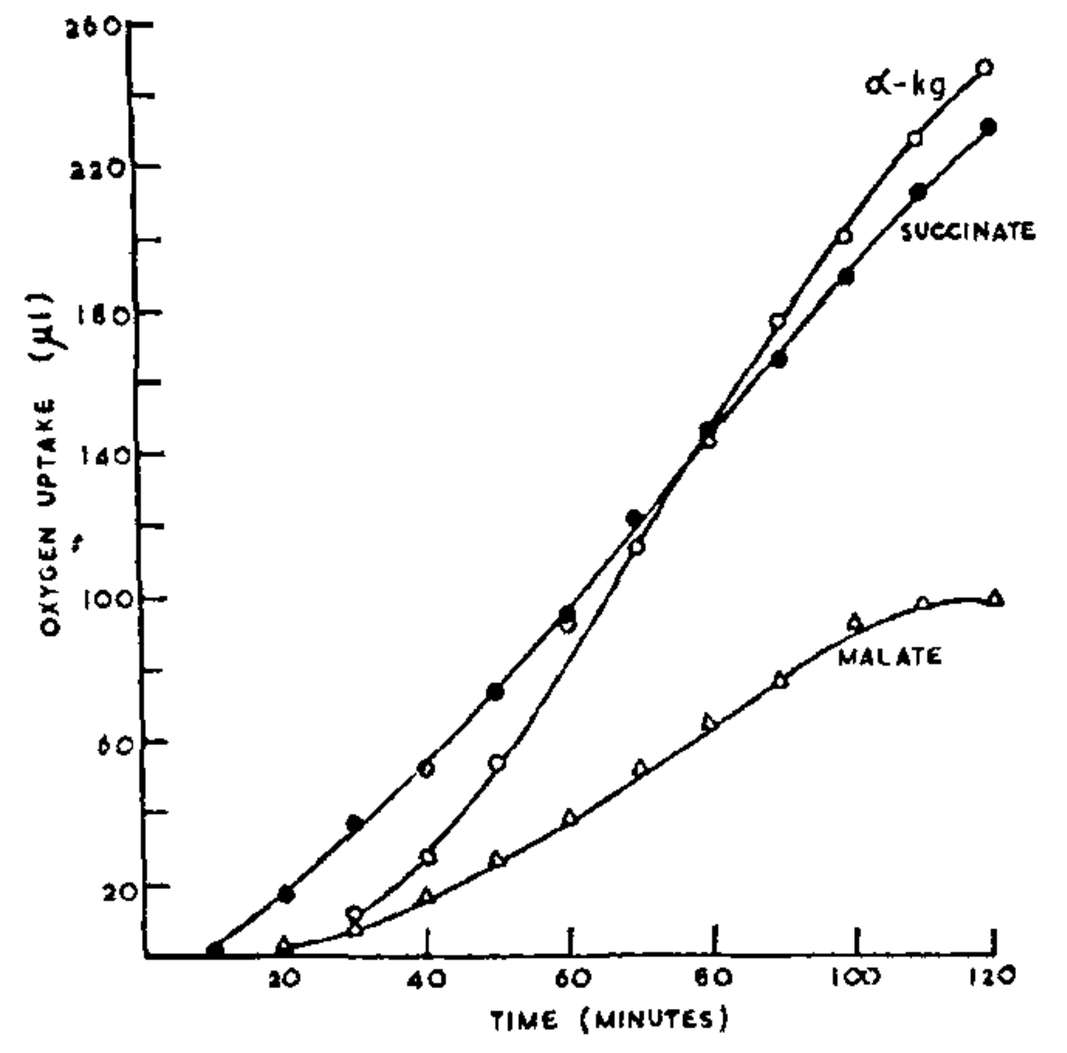

FIG. 2 *b*. Oxygen uptake from the oxidation of α-kg, succinate and malate by washed cells of *Arthrobacter* strain C_{19}d grown on glucose.

transaminase and glutamic dehydrogenase as proposed by Braunstein and Asarkh[15], which can give rise to an overall reaction leading to alanine accumulation, is also discounted in this organism for the following reasons. The presence of catalytic amounts of either α-ketoglutarate or glutamate would allow this overall reaction to proceed. But in the present studies, the low activities of glutamic dehydrogenase and α-ketoglutarate-alanine transaminase found in the cell-free extract of the organism cannot account for the accumulation of such large quantities of alanine in the growth medium. The activity of alanine dehydrogenase in this organism being very high, the coupled system of these enzymes proposed by the above authors, if active in this organism, may only play a minor role.

Samejimia *et al.*[16] in their studies with an alanine accumulating bacterium (which they designated as strain 483) demonstrated very high L-alanine dehydrogenase activity in the cell-free extracts of the organism. The enzyme was found to be specific for NADH in its co-factor requirement. Alanine formation by transamination was found to be negligible, whereas transamination between α-ketoglutarate and alanine was absent. So the authors concluded reductive amination of pyruvate as the main route of alanine synthesis. Findings here are almost similar except for the transamination between α-ketoglutarate and alanine, which was noticed to a small degree. However, the activity of this transaminase was very low and moreover it was found that the α-ketoglutarate supply for this reaction is limited since α-ketoglutarate dehydrogenase activity is quite high. Hence the utilization of accumulated alanine for this reaction will be negligible. This observation is in accordance with the results obtained from cultural experiments, where very little glutamic acid accumulation was noticed under conditions favourable for alanine accumulation.

The results obtained from the cell-free extract studies confirm the observations made with labelled compounds and with the metabolic inhibitor. Results seem to point out that alanine biosynthesis in this organism is mainly through reductive amination of pyruvate catalysed by alanine dehydrogenase.

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