Pyruvate Metabolism by a Nitrogen-Fixing Bacterium

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1. The major products of pyruvate dissimilation by washed intact cells of *Achromobacter* N4-B under nitrogen-fixing conditions are acetate and formate. The formation of succinate and isocitrate and the assimilated amino acids requires carbon dioxide fixation. 2. The products formed by cells incubated with pyruvate in an atmosphere of nitrogen were compared with those formed by cells incubated in an atmosphere of helium. Production of hydrogen and the formation of succinate were greater under helium than under nitrogen. Production of acetate and formate and the utilization of pyruvate were the same in both atmospheres. 3. Cell-free preparations, unlike intact cells of *Achromobacter* N4-B, do not evolve hydrogen, but do produce lactate. 4. It is suggested that, in cell-free preparations incapable of fixing nitrogen, electrons are accepted from pyruvate to form lactate rather than being used for the reductive formation of ammonia and hydrogen.

The dependence of nitrogen fixation by *Achromobacter* N4-B on the metabolism of pyruvate was reported previously (Hamilton, 1962; Hamilton, Burris & Wilson, 1964). Because pyruvate was as effective as mannitol in supporting fixation it appeared that the metabolic reactions of mannitol before pyruvate was formed were not involved directly in the process of nitrogen reduction to ammonia. Only pyruvate and α -oxobutyrate are effective energy sources for nitrogen fixation with cell-free preparations of *Clostridium pasteurianum* (Carnahan, Mortenson, Mower & Castle, 1960). Pyruvate also supports the fixation of nitrogen by *Bacillus polymyxa* (Grau & Wilson, 1962, 1963) and by *Aerobacter aerogenes* (Lindsay & Wilson, 1963).

The products of pyruvate metabolism by intact cells and by extracts that contained no fixing activity have been studied. The results provide an insight into the reactions of pyruvate dissimilation associated with the nitrogen-fixing process.

MATERIALS AND METHODS

Organism. *Achromobacter* N4, received from Jensen (Jensen, 1958), was the parent strain used in this work. A stable non-gummy variant of this organism was obtained by serial selection of nitrogen-grown cells and used in all of the experiments reported below; this strain was designated *Achromobacter* N4-B.

Growth. *Achromobacter* N4-B was maintained aerobically on nutrient-agar slopes, subcultured monthly, incubated at 30° and stored at 4°. Before studies of nitrogen fixation, the organism was transferred in the nitrogen-free medium of Goerz & Pengra (1961) three to four times in an atmosphere of nitrogen. The carbon source for growth was

mannitol (2%, w/v), as less extracellular polysaccharide was formed by the fixing cells with this energy source than with glucose, sucrose and a variety of other carbohydrates (I. R. Hamilton, unpublished work).

A limiting amount of combined nitrogen (20 μ g. of nitrogen/ml.) was always incorporated into the medium to permit rapid initial growth. Combined nitrogen is always used preferentially to elemental nitrogen (Goerz & Pengra, 1961) and allows the cells to utilize the traces of oxygen remaining in the growing flasks after gassing with nitrogen. After the complete utilization of the NH_4^+ nitrogen, a lag period lasting 6–12 hr. occurs before growth resumes on nitrogen gas. This lag in the biphasic growth of the organism appears to represent the period of induction for the enzyme or enzymes which reduce nitrogen to ammonia.

Small cultures were always grown in 100 ml. of liquid medium in 500 ml. flasks stopped with a one-hole rubber plug. The nitrogen atmosphere was obtained by evacuating the flasks and gassing five to seven times with commercial nitrogen containing less than 0.01% of impurities. The flasks were then incubated at 30° on a rotating shaker (New Brunswick Co., New Brunswick, N.J., U.S.A.) at 250 rev./min. Growth was followed by measuring turbidity in a Klett-Summerson photoelectric colorimeter with a 640–700 $m\mu$ filter and a side arm on the growth flasks consisting of a Pyrex tube that fitted the holder of the colorimeter. Since turbidity was directly proportional to total cell nitrogen as measured by a micro-Kjeldahl method, cell and extract concentrations are expressed as mg. of total nitrogen.

For larger quantities, cells were grown in 1400 ml. of medium in suction flasks (21.) fitted with a three-hole stopper for gassing, pH adjustment and sampling. Sampling was carried out by connecting the sampling tube to a 50 ml. suction flask, which in turn was connected to a vacuum source. A vacuum was drawn on the small flask after which the screw clamp on the sampling tube was opened slowly, allowing 10–15 ml. of culture suspension to enter the small flask. Turbidity and the pH were measured on this sample.

The use of sampling flasks in this manner was necessitated to prevent changing the gas phase above the growing culture (Hamilton *et al.* 1964). The pH of the medium was adjusted with sterile 2N-NaOH added through a separatory funnel by gentle pressure, care being taken not to allow air into the gas phase. These flasks were incubated on a rotary shaker at 30° as previously described.

Harvesting. Where large quantities of cells were collected, the Sorvall Szent-Gyorgyi and Blum continuous-flow apparatus (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) was employed with a SS-1 centrifuge. Cells were collected anaerobically by this method, at fields up to 30000g, in one or two 45 ml. tubes, depending on the quantities. The cells were thus kept under anaerobic conditions until washing. All buffers used for washing and subsequent treatments were degassed by heating, followed by flushing at 0° with nitrogen or hydrogen. All manipulations with intact cells and extracts were carried out in a stream of nitrogen or hydrogen.

Preparation of extracts. Cell-free preparations were made by exposing washed intact cells of *Achromobacter* N4-B to either ultrasonic or mechanical breakage. Ultrasonic breakage was accomplished with a 20 kcyc./sec. Mullard ultrasonic oscillator equipped with a titanium probe (Instrument Associates, New York, N.Y., U.S.A.) by treating cell suspensions (5–30 ml.) for 10–20 min., depending on the volumes. During exposure, the cells were agitated in a jacketed glass chamber with a small magnetic stirring bar at or near 4°. The French pressure cell (American Instrument Co., Silver Spring, Md., U.S.A.) was used for mechanical breakage; two passages were necessary to obtain suitable preparations. In both cases, the supernatant fraction from centrifugation at 28000g for 30 min. was called the cell-free preparation.

Procedures for pyruvate dissimilation

Whole cells. Sodium pyruvate (45–167 mm), obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., was incubated with washed intact cells of *Achromobacter* N4-B, varying in concentration from 0.65 to 1.57 mg. of total cell nitrogen/ml., in sodium phosphate buffer (0.1M; pH 7.5) in a gas phase of nitrogen or helium. Products were analysed in two ways: (1) both the gaseous and non-gaseous products were analysed from the same reaction mixture in a volume of 3.0 ml. in Warburg flasks; the reaction was stopped by the addition of 0.3 ml. of 10N-H₂SO₄ followed by titration to pH 7.5 with N-NaOH and centrifugation to remove cells; (2) gas analysis was carried out in a 2.4 ml. volume by a triple-side-arm technique, and non-gaseous product formation was followed in 20.0 ml. volumes with the same reaction mixture. The large reactions were stopped by the addition of 1.5 ml. of 25% (w/v) ZnSO₄ and a 4.2 ml. sample was immediately taken for acetyl phosphate determination. The remainder of the mixtures were then titrated to pH 7.7 with N-NaOH and the cells centrifuged from the solution. The supernatant was diluted to 50 ml., filtered through a 0.45 μ Millipore filter and stored at –20°.

The assimilation of pyruvate was followed with intact cells separately with the use of [3-¹⁴C]pyruvate (Volk Radiochemical Co.) according to the methods previously described (Hamilton *et al.* 1964).

Cell-free extracts. The supernatant fraction after cell breakage was incubated with 20 mm-sodium pyruvate in sodium phosphate buffer (0.1M; pH 7.5) containing 2-

mercaptoethanol (1 mm); the extract concentration was 1.62 mg. of total nitrogen/ml. Reactions were carried out in volumes of 3.0 ml. with gas evolution and non-gaseous product formation was followed in the same flask according to the methods outlined above. All reactions were performed anaerobically at 30°.

Assay procedures

Gas evolution. Hydrogen and carbon dioxide evolution were determined manometrically by the direct method (Umbreit, Burris & Stauffer, 1964) and by a triple-side-arm technique. The latter technique permitted the determination of evolution of both carbon dioxide and hydrogen in a single flask and was found to be more accurate than separate flasks for each gas. The flask employed had two side arms: one of these was equipped with a double sac containing 0.3 ml. of 12N-H₂SO₄ and 20% (w/v) freshly prepared KOH, and the other was a normal side arm containing substrate, or buffer in the endogenous controls. Gassing was always carried out by forming a vacuum on both sides of the manometers and filling the manometers and flasks with the required gas. This process was repeated four to six times to ensure anaerobic conditions.

After equilibration of the flask at 30° for 10–20 min., the substrate was tipped, care being taken not to mix or tip in the contents of the double sac. The reaction was stopped by the addition of the acid into the main chamber in such a way that the KOH remained in the side arm; this was followed by equilibrating the flasks until no further pressure changes were observed. The reading taken at this point and corrected for changes in temperature and pressure constituted the hydrogen evolved, provided that the change was greater than the endogenous control run at the same time. Addition of the KOH into the main chamber released the absorbed carbon dioxide, as the acid concentration was sufficient to reduce the pH below 2. After a further equilibration, a final reading was taken and represented the total gas evolved. The net difference between the reading after tipping in the acid and that after the release of the carbon dioxide represented the carbon dioxide evolved.

Non-gaseous products. Short-chain fatty acids were analysed by the method of Bulen, Varner & Burrell (1952) with a gradient elution technique similar to that of Bock & Ling (1954). Rapidly flowing columns were prepared with 100-mesh chromatography silicic acid minus fines. Such columns were prepared by thoroughly mixing 3.5 ml. of 0.5N-H₂SO₄ with 5g. of silicic acid; this was mixed with chloroform, also equilibrated with 0.5N-H₂SO₄, then poured and gently packed into columns. A filter disk was placed on top, and above this was added 1g. of non-acid-equilibrated silicic acid in chloroform; the column was usually 13 cm. $\times$ 1.3 cm.². The non-equilibrated silicic acid dehydrated the aqueous samples so that the acids would be mobile in the organic solvent system. The columns were eluted with a gradient of up to 20% (v/v) butan-1-ol in chloroform; the solvents were equilibrated with 0.5N-H₂SO₄. Fractions of volume 2.5 ml. were collected and titrated with ethanolic 0.2–10 mM-NaOH.

With this method it was possible to separate acetic acid, formic acid and pyruvic acid, but lactic acid and succinic acid were eluted in the same fractions. The amount of succinic acid was determined by calculating the difference between the amount of acid titrated and the amount of

lactic acid assayed colorimetrically by the method of Barker & Summerson (1941).

The non-volatile organic acids were separated on Dowex 1 (X8; formate form; 100–200 mesh; Dow Chemical Co., Midland, Mich., U.S.A.) according to the method of Palmer (1955). Organic acids were separated and identified by paper chromatography by the methods of Palmer (1955) and Smith (1960).

Pyruvic acid was determined by a modification of the method of Friedemann & Haugen (1943). The filtered reaction mixtures were not extracted with ethyl acetate after the addition of 2,4-dinitrophenylhydrazine, but the samples were developed directly with 2*N*-NaOH and read at 520 m μ . Acetoin plus diacetyl were assayed by the method of Westergaard (1945), and diacetyl by the method of White, Krampitz & Werkman (1946). Acetyl phosphate was measured by the method of Stadtman & Barker (1950), and the formic acid eluted from silicic acid columns was confirmed by the method of Grant (1948).

RESULTS

Intact organisms. The optimum conditions for nitrogen fixation by intact washed cells of *Achromobacter* N4-B have been established by the sensitive

¹⁵N technique of Burris & Wilson (1957). Maximum fixation occurred at 30° with 180 mm-sodium pyruvate in 0.1 M-sodium phosphate buffer, pH 7.5, in an atmosphere of ¹⁵N₂ and helium (Hamilton, 1962). When pyruvate is metabolized under these conditions, the pH of the reaction mixture falls considerably. Initial analyses of the reaction mixtures indicated acetate and formate to be the major products of pyruvate degradation; more acetate than formate was formed, and the hydrogen and carbon dioxide evolved were not equimolar (Hamilton, 1962). It thus appears that two types of pyruvate cleavage occur under these conditions: (1) the cleavage to acetate and formate and (2) degradation to acetate, carbon dioxide and hydrogen.

Further investigation of the products showed that small amounts of succinate and isocitrate accumulated in the medium, suggesting that carbon dioxide was fixed during the dissimilation of pyruvate. Carbon dioxide fixation was confirmed by incubating cells of *Achromobacter* N4-B with pyruvate and [¹⁴C]bicarbonate (prepared from barium [¹⁴C]-carbonate obtained from Oak Ridge National

Table 1. *Anaerobic dissimilation of sodium pyruvate by washed cells of Achromobacter N4-B under optimum nitrogen-fixing conditions*

Nitrogen-grown cells of *Achromobacter* N4-B, at a concentration of 0.65 mg. of total nitrogen/ml., were incubated with 45 mm-sodium pyruvate in a reaction volume of 20 ml. in 75 ml. Warburg flasks. Values were determined after 3 hr. at 30°, which was preceded by a 30 min. equilibration period. Gas evolution was determined in separate 2.4 ml. reaction volumes of identical composition. The gas phase was nitrogen.

Compound	μ moles/100 μ moles of pyruvate	μ moles of carbon	Microequivalents	
			Oxidation	Reduction
Sodium pyruvate	100	300	—	200
Nitrogen fixed	2.9	—	17.4	—
Acetate	85.0	170.0	—	—
Formate	72.0	72.0	144.0	—
Isocitrate	3.0	18.0	18.0	—
Succinate	1.2	4.8	2.4	—
Carbon dioxide	7.8	7.8	31.2	—
Hydrogen	12.5* calc. 0.9 obs.	—	—	25.0
Assimilated aspartate†	3.3	13.2	13.2	—
		285.8	226.2	225.0

Carbon recovery = 95.3%. $O/R\ddagger = 1.01$.

* See Table 2 for the determination of the true hydrogen evolved during the reaction.

† Assimilated refers to products of pyruvate metabolism bound into cellular constituents. The oxidation value for aspartic acid is normally + 2; however, it is considered here as + 4, which would be the value if the amino group were hydrolysed away. The amino group is considered to have arisen anew from nitrogen fixation.

‡ O/R is the ratio of oxidized to reduced products. As the O/R ratio is calculated to express the degree of oxidation and reduction compared with glucose ($O/R = 0$), then substrates having oxidation values greater than or less than zero are placed in the opposite column to that indicated by the sign. Thus pyruvate ($O/R = + 2$) is placed in the reduction column instead of the oxidation column, as indicated by the sign. If, on the other hand, glycerol had been fermented ($O/R = - 2$), then glycerol would have been placed in the oxidation column.

Laboratory) as in the experiments described by Hamilton, Burris, Wilson & Wang (1965). Their observation that a small amount of the pyruvate carbon is assimilated by the cells during the reaction period was confirmed by radio-respirometric experiments with specifically labelled pyruvate. The label from [^{14}C]bicarbonate incubated with pyruvate and cells of *Achromobacter* N4-B also appeared in eight ninhydrin-positive cellular components, primarily in aspartate and glutamate. As aspartate constitutes the largest fraction of the assimilated products, and as the pathways for biosynthesis of the amino acids in this organism are not known, calculation of the fermentation balances was simplified by considering this amino acid as the only product incorporated into the cells. As little pyruvate carbon is assimilated, the overall error from this simplification will be small.

Table 1 shows the fermentation balance for pyruvate degradation with washed whole cells of *Achromobacter* N4-B under optimum nitrogen-fixing conditions. The values have been calculated on the basis of 100 μmoles of pyruvate for convenience. For the determination of the oxidation-reduction state of the substrate and products in the fermentation balance calculations, the hydrogen atom is given a value -1 and the oxygen atom a value $+2$ (i.e., $\text{H}\cdot\text{CO}_2\text{H}$ has an oxidation-reduction value $+2$). The determinations for isocitrate and the assimilated product aspartate were obtained from a separate experiment with [$3\text{-}^{14}\text{C}$]pyruvate under identical conditions.

The major products of dissimilation are the volatile acids, acetic acid and formic acid, in non-equimolar amounts. The remaining products, although small in quantity, contribute significantly to the oxidation portion of the balance. The influence of carbon dioxide fixation is apparent in the appearance of succinate and aspartate, in addition to isocitrate. Fig. 1 illustrates the time-course of pyruvate degradation and product formation. The formation of the products, except for isocitrate (Fig. 1), generally coincides with the utilization of pyruvate. Formation of aspartate and succinate, and the fixation of nitrogen, cease with the exhaustion of pyruvate.

The uptake of evolved carbon dioxide occurs in the absence of pyruvate; at the same time isocitrate increases slightly. Acetate and formate decrease in the absence of pyruvate, but it is not known whether the decrease of these products together with carbon dioxide supports the re-formation of pyruvate (reversal of the phosphoroclastic reaction) followed by further biosynthesis via the citric acid cycle.

The calculation of hydrogen evolution under these conditions must allow for hydrogen used in the reduction of nitrogen, as Hamilton *et al.* (1964) have shown that hydrogen evolution is greatly reduced

Fig. 1. Time-course of product formation during pyruvate metabolism with washed cells of *Achromobacter* N4-B under optimum nitrogen-fixing conditions. Conditions are described in Table 1. O, Carbon dioxide evolution; ▲, calculated hydrogen evolution; ■, total nitrogen; Δ, aspartic acid; ●, isocitric acid; □, succinic acid; x, acetic acid; ◻, formic acid; ○, residual pyruvate.

in the presence of nitrogen gas and nitrogen-fixing cells furnished with adequate pyruvate. In this experiment, only 0.9 μmole of hydrogen was actually detected, and 5.8 μmoles of nitrogen were fixed. Table 2 outlines the calculations, which indicate that 12.5 μmoles of hydrogen would have been evolved had no nitrogen been fixed. The ammonia formed can be metabolized in a variety of ways; 2.2 μmoles of combined nitrogen were detected in the bacterial filtrate at the end of the reaction period. The amount of ammonium nitrogen formed and assimilated into cellular material (3.6 μmoles) is assumed to have formed the amino group of the aspartate, and it is assumed that 'ammonia pools' in organisms fixing nitrogen are low compared with those in cells growing on a combined source of nitrogen.

The list of compounds formed does not include lactate, as it does not accumulate and is involved only to a minor extent in the reactions. Lactate (2 μmoles) was formed immediately after the addition of pyruvate and then disappeared. The concentration of cellular NADH_2 may have been adequate to reduce some of the pyruvate to lactate

Table 2. *Calculation of the true hydrogen evolution during nitrogen fixation*

	$\mu\text{moles}/100\mu\text{moles}$ of pyruvate
Net nitrogen fixed in 3 hr.	2.9
Ammonia formed during fixation	5.8
Hydrogen required to reduce 2.9 μmoles of nitrogen	8.7
Hydrogen actually detected	0.9
Hydrogen not detected due to uptake of N_2 gas (loss of gas volume)	2.9
Hydrogen for reduction	8.7
True hydrogen evolved with no fixation	12.5
Kjeldahl nitrogen in filtrate	2.2
Cellular combined nitrogen increase	3.6
Total combined nitrogen as ammonia	5.8

Table 3. *Comparison of products from the metabolism of pyruvate by washed cells of Achromobacter N4-B in atmospheres of helium and nitrogen*

Cells were incubated at a concentration of 1.08 mg. of total nitrogen/ml. for 3 hr. at 30°. All analyses were carried out with the same reaction mixture in a volume of 3.0 ml.

	$\mu\text{moles}/\text{ml. of reaction mixture}$	
	In helium	In nitrogen
Initial pyruvate	167.0	167.0
Pyruvate utilized in 180 min.	121.0	119.0
Acetate	76.0	73.5
Formate	39.0	40.0
Succinate	6.7	2.8
Diacetyl	1.2	1.6
Carbon dioxide	11.0	13.7
Hydrogen	6.1	3.8

during the 30 sec. period between the addition of pyruvate and the quenching of the zero-time samples. It is postulated that the electron flow would shift to the hydrogen- and nitrogen-activating systems after the initial lag sometimes observed in hydrogen evolution. Formation of lactate would account for 2 μmoles of acetate and 2 μmoles of carbon dioxide by the dismutation reaction of Korkes, Del Campillo, Gunsalus & Ochoa (1951). Lactate could be converted into 2 μmoles of pyruvate when sufficient NAD accumulated to reverse the reaction.

Very little acetyl phosphate was detected despite immediate assay for this product after the reactions were quenched. Acetyl-CoA may be converted into acetate via acetyl phosphate so rapidly that this intermediate is not detected.

Washed cells of *Achromobacter* N4-B, grown on nitrogen and incubated with 167 mM-pyruvate, released less hydrogen and more carbon dioxide in an atmosphere of nitrogen than in one of helium (Table 3). This increase in carbon dioxide in a nitrogen atmosphere generally was not evident at lower pyruvate concentrations. More succinate accumulates in helium than in nitrogen, possibly because the formation of amino acids is reduced in the absence of nitrogen. This high concentration of pyruvate supported some diacetyl accumulation, but the major products, such as acetate and formate, were produced and pyruvate was utilized to about the same extent in the two gas systems.

Cell-free extracts. Studies with intact cells and cell fractions of nitrogen-grown *Achromobacter* N4-B have shown that both the pyruvate-dependent nitrogen-fixing and hydrogen-evolving activities are particulate, being associated with a cell wall-membrane fraction (Hamilton *et al.* 1964). Cell-free

extracts, free from the particulate material, had no hydrogenase or nitrogenase activity, but did decarboxylate pyruvate; the particulate fraction had no decarboxylating activity.

As Table 4 illustrates, the only differences between the products of pyruvate metabolism with intact nitrogen-fixing cells (Table 1) and non-fixing cell-free preparations are the decrease in hydrogen evolved and the formation of lactate by the extract material. The major products of pyruvate disimilation continue to be acetate and formate, and carbon dioxide-fixation products (isocitrate and succinate) appear in increased concentrations, probably because no amino acid synthesis occurs in the absence of nitrogen fixation as it does with intact cells.

The formation of carbon dioxide with these extracts, free of hydrogenase, occurs via the dismutation reaction, in which 2 moles of pyruvate are degraded to 1 mole each of acetate, lactate and carbon dioxide (Korkes *et al.* 1951).

The shift in product formation between cells and extracts is further shown by nitrogen-grown cells (used to prepare the extracts for the experiment in Table 4) when incubated with pyruvate under the same conditions as used for the extracts, at approximately the same total nitrogen concentration. During the same incubation period, the intact cells failed to produce lactic acid; in fact, 0.5 μmole (per 100 μmoles of pyruvate) of the initial endogenous lactic acid was degraded. As is shown in Table 4, the extracts produced 13.7 μmoles of lactic acid from 100 μmoles of initial pyruvate in the same time.

As with intact cells, some diacetyl formation is observed if the pyruvate concentration is increased; 2.7 mM-diacetyl was produced by increasing the pyruvate concentration from 20 mM to 33 mM and

Table 4. *Anaerobic dissimilation of sodium pyruvate by cell-free extracts of Achromobacter N4-B*

The cell-free extract, at a concentration of 1.62 mg. of total nitrogen/ml., was incubated with 20 mM-sodium pyruvate in a total reaction volume of 3.0 ml. at 30° for 3 hr. The gas phase was nitrogen. All analyses were carried out with the same reaction mixture.

Compound	$\mu\text{moles}/100\mu\text{moles}$ of pyruvate	μmoles of carbon	Microequivalents	
			Oxidation	Reduction
Sodium pyruvate	100	300	—	200
Acetate	63.8	127.6	—	—
Formate	58.2	58.2	116.4	—
Lactate	13.7	41.1	—	—
Succinate	6.9	27.6	13.8	—
Isocitrate	6.0	36.0	36.0	—
Carbon dioxide	6.9	6.9	27.6	—
Hydrogen	0.1	—	—	0.3
		297.4	193.8	200.3

Carbon recovery = 99.1%. $O/R^* = 0.97$.

* See Table 1.

adding 0.7 mM-thiamine pyrophosphate and 14 mM-magnesium chloride. Under these conditions the formation of this reduced product was balanced by an increased evolution of carbon dioxide. The formation of diacetyl can be greatly enhanced by lowering the pH of the reaction to 6.5. At this pH, diacetyl and carbon dioxide are the major products of the degradation of pyruvate, while acetate and formate appear but in smaller quantities. Carbon dioxide fixation and the formation of lactate still occur at this pH as they do at pH 7.5.

DISCUSSION

Although product analyses afford limited information on individual degradative reactions, comparisons between nitrogen-fixing and non-fixing preparations are valuable in illustrating fundamental metabolic differences. It is evident that the dissimilation of pyruvate by nitrogen-fixing intact cells of *Achromobacter* N4-B is complex. In addition to demonstrable nitrogen-fixing, the fixation of carbon dioxide and the assimilation of pyruvate carbon have been observed. The demonstration that, when pyruvate is the carbon source, the label from [^{14}C]-bicarbonate can be found in intracellular amino acids, which in turn appear in the cellular protein fraction, illustrates the importance of pyruvate in the metabolism of this organism (Hamilton *et al.* 1965). Although this organism exhibits citric acid-cycle activity aerobically (I. R. Hamilton, unpublished work), the cycle appears to be used only for biosynthetic purposes under anaerobic conditions.

Apart from providing the building blocks for essential biosynthetic reactions, pyruvate must provide all the necessary energy for the reduction of nitrogen to ammonia. With *Achromobacter* N4-B, the flow of electrons originating with pyruvate

appears to be controlled by the gaseous environment in the absence of a combined nitrogen source. No hydrogen is evolved from pyruvate by ammonia-grown cells in atmospheres of nitrogen, hydrogen or helium. Nitrogen-grown cells, which normally exhibit a weak hydrogenase (Hamilton *et al.* 1964), lose the ability to form hydrogen on preincubation with small amounts of ammonium salts.

The low activity of hydrogenase in *Achromobacter* N4-B has permitted the demonstration of nitrogen inhibition of hydrogen evolution (Hamilton *et al.* 1964). This was observed by carrying out identical incubations with cells and pyruvate in nitrogen and in helium. It can be postulated therefore that, as the reduction of nitrogen to ammonia is an essential reaction for the cell, the electron flow is preferentially in the direction of nitrogen activation. The reduction could utilize electrons via hydrogenase, as has been shown with cell-free preparations of *Clostridium pasteurianum* (D'Eustachio & Hardy, 1964). In the absence of nitrogen, electrons would activate protons with the formation of hydrogen gas.

Previous studies have shown that nitrogen-fixing and hydrogenase activities are membrane-bound (Hamilton *et al.* 1964). Significantly, particle-free extracts of nitrogen-grown cells of *Achromobacter* N4-B, containing no hydrogenase or nitrogenase activity, divert electrons from pyruvate to the lactate-dehydrogenase system, resulting in the formation of lactic acid. Scheme 1 illustrates the electron flow for the various preparations incubated with pyruvate anaerobically. Whether NAD constitutes a component of the electron-transport system of this organism is unknown. Although ferredoxin (Mortenson, Valentine & Carnahan, 1962) has not been isolated from the facultative anaerobic nitrogen-fixing organisms, a low-redox-potential electron