Electron Transport Carriers Involved in Nitrogen Fixation by the Coliform, *Klebsiella pneumoniae*

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SUMMARY

In cell-free extracts, pyruvate, formate, malate and NADPH provided reducing equivalents to the nitrogenase of the nitrogen-fixing coliform, *Klebsiella pneumoniae*. Two electron carriers were isolated from extracts, both capable of mediating the transfer of reducing power between illuminated spinach chloroplasts and the nitrogenases of *Klebsiella* or *Azotobacter vinelandii*. One electron carrier was a flavoprotein, named *Klebsiella* flavodoxin (molecular weight about 21 000); the second was not characterized. A similarity is suggested between *Klebsiella* and *Escherichia coli* in the generation and transport of low-potential reducing power from pyruvate.

INTRODUCTION

*Klebsiella pneumoniae** is a coliform bacterium that is capable of nitrogen fixation under anaerobic (Hamilton & Wilson, 1955) or microaerophilic (Klucas, 1972) conditions and is commonly isolated from plant and soil sources (Silver, Centifanto & Nicholas, 1963; Pengra, 1964; Mahl, Wilson, Fife & Ewing, 1965). The metabolism (Hamilton & Wilson, 1955) and genetic make-up (Matsumoto & Tazaki, 1970; Streicher, Gurney & Valentine, 1971; Dixon & Postgate, 1971, 1972) of *K. pneumoniae* show it to be closely related to *Escherichia coli*. Although the nitrogenase of *Klebsiella* has been studied extensively by Biggins & Kelly (1970) and Eady, Smith, Cook & Postgate (1972), little is known of the electron transport pathway that couples the oxidation of substrate to the reduction of the nitrogenase enzyme. In other nitrogen-fixing organisms, ferredoxins and flavodoxins transfer highly reducing electrons to nitrogenase (see review by Burris, 1971). Sodium dithionite (Mahl & Wilson, 1968) or hydrogenase-reduced methyl viologen (Eady *et al.* 1972) (both non-physiological reductants) have until now been the only reductants found to provide reducing power for nitrogen fixation in cell-free extracts of *Klebsiella*.

An artificial reductant which has proven useful in elucidating the electron transport systems of *Chromatium* (Yoch & Arnon, 1970), *Azotobacter vinelandii* (Benemann, Yoch, Valentine & Arnon, 1969; Yoch, Benemann, Valentine & Arnon, 1969), soybean root nodule bacteroids (Yoch *et al.* 1970; Koch *et al.* 1970) and *Bacillus polymyxa* (Yoch, 1973), is illuminated chloroplasts. Unlike dithionite, which reduces the nitrogenase directly (Bulen, Burns & LeComte, 1965), chloroplasts reduce nitrogenase only through intermediate low-potential electron carriers such as ferredoxin (Fd) or flavodoxin (Fld) (reaction 1).

* On the basis of serological and biochemical tests, Mahl, Wilson, Fife & Ewing (1965) reclassified various nitrogen-fixing strains of *Klebsiella*, *Aerobacter aerogenes* and *Achromobacter* as *Klebsiella pneumoniae*.

This 'chloroplast-nitrogenase' technique has now been used to identify in *Klebsiella* two electron transport components capable of transferring low-potential reducing power to the nitrogenase system.

In addition to reporting the partial purification and biochemical activities of the two low-potential electron transport components from *Klebsiella*, this paper reports the physiological substrates that are capable of providing reducing power of *Klebsiella* nitrogenase in cell-free extracts and the activity of *E. coli* flavodoxin and a pyruvate dehydrogenase system from *E. coli* in coupling to *Klebsiella* nitrogenase.

METHODS

Preparation of bacterial extracts. *Klebsiella pneumoniae* strain M5al, provided by P. W. Wilson from the culture collection of the University of Wisconsin, was cultured in 12 l carboys on a mineral salts medium as described by Yoch & Pengra (1966). Nitrogen was provided by sparging the culture with nitrogen gas. *Klebsiella* extracts were prepared by disrupting frozen cell paste in a Hughes press (other methods of cell disruption proved to be less satisfactory, especially for pyruvate- and formate-supported nitrogenase activity). The resulting frozen extract was thawed in 1 vol. 0.02 M-N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES) buffer, pH 7.4, plus 1 mg deoxyribonuclease under an atmosphere of argon. After incubation with deoxyribonuclease for 30 min at room temperature, the extract was centrifuged at 35 000 g for 15 min and the supernatant was retained as the *Klebsiella* nitrogenase extract. When the extract was stored under an atmosphere of argon at -20 °C, dithionite-supported nitrogenase activity was stable (that is, it retained more than 50 % of its original activity) for 8 to 10 months.

The endogenous electron carriers were removed by passing *Klebsiella* extracts (approx. 10 ml portions) that had been stored at -20 °C for a month or more over a 2 × 3 cm DEAE-cellulose column which had previously been equilibrated with argon-saturated 0.02 M-HEPES buffer, pH 7.4. As a column of this size was quickly saturated with protein, only the more acidic proteins, such as the *Klebsiella* electron carriers, were bound to the DEAE-cellulose column. The more weakly acidic nitrogenase proteins were not retained by the column and were recovered in the effluent (as measured by C₂H₂-reducing activity, over 90 % of the nitrogenase passed through the column). After washing the DEAE-cellulose column with 10 to 15 ml HEPES buffer, the electron carriers were eluted with buffer containing 0.8 M-NaCl. For washing the column and eluting the electron carriers, anaerobic precautions were not necessary as these carriers were stable in air.

Repeated freeze-thaw cycles and various detergent treatments were tried, but only prolonged freezing followed by DEAE-cellulose treatment removed the electron carriers to the extent that the nitrogenase could be used to assay the isolated electron carriers in the chloroplast-nitrogenase system (reaction 1). Because the period of freezing could not be standardized, not all extracts frozen and treated on DEAE-cellulose could be used to assay for electron carriers. A dilemma arises in that methods and reagents drastic enough to release the electron carriers also destroy the nitrogenase. The prolonged freezing of the *Klebsiella* extract is the only known method that will release the electron carriers while leaving a functional nitrogenase enzyme.

Azotobacter vinelandii was cultured, harvested and disrupted as previously described by Benemann *et al.* (1969). Nitrogenase extracts were prepared and the endogenous electron carriers were removed by treatment on a DEAE-cellulose column as previously described by Benemann, Yoch, Valentine & Arnon (1971).

Escherichia coli, Crookes strain, was cultured anaerobically on L-broth (a glucose-yeast extract-tryptone medium) described by Luria & Burrous (1957). Frozen cells were disrupted in a Hughes press as described above for *Klebsiella*. The supernatant extract (35000 g for 10 min) was stored under argon and used within several hours of preparation.

Purification of the Klebsiella electron carriers. To purify the electron carrier component from nitrogen-grown *Klebsiella* cells, 500 g cell paste was suspended in 2 vol. 0.02 M-phosphate buffer, pH 7.4 (hereafter referred to as the buffer). The cell suspension was sonicated for 5 min with a 20 kHz Branson sonifier at full power. The extract was brought to 30 % (v/v) with cold (-20°C) *n*-butanol and was constantly stirred for 30 min at 5°C . The butanol extract was centrifuged for 30 min at 10000 g and the resulting butanol-containing supernatant solution was passed over a DEAE-cellulose column. After the column was washed with several volumes of buffer, the yellowish-brown band that had collected at the top of the column was eluted with buffer containing 0.5 M-NaCl and dialysed against buffer overnight. This solution was placed on a DEAE-cellulose chromatography column (1.5×45 cm) previously equilibrated with buffer. The column was developed with 150 ml buffer containing 0.1 M-NaCl, followed by buffer containing 0.2 M- and then 0.32 M-NaCl. Four coloured bands eluted from this column; each was further purified by Sephadex G-50 column chromatography. The characterization and biological activities of these fractions are discussed in Results.

Assay of enzymic activities. The *Klebsiella* electron carrier fractions were assayed for their ability to mediate electron transport between illuminated spinach chloroplast fragments (prepared as described by Yoch, 1972) and either *Klebsiella* or *Azotobacter* nitrogenase. The conditions of the assay and the content of the reaction mixture are described in the legend to Fig. 1. Nitrogenase activity was measured by the reduction of acetylene to ethylene (Schöllhorn & Burris, 1966; Dilworth, 1966) as determined by gas chromatography (Hardy, Holsten, Jackson & Burns, 1968).

The *Klebsiella* electron carriers were also assayed for their ability to substitute for plant ferredoxin in the photochemical reduction of NADP^{+} using either heated (55°C , 5 min) washed chloroplasts or blue-green algae particles. The reaction mixture was that described for Fig. 1 of Yoch *et al.* (1969). The details of light source, filters, and geometry of the system for continuous monitoring of NADP^{+} reduction were described by McSwain & Arnon (1968).

Electron carriers. *Escherichia coli* flavodoxin, a gift of J. Knappe, was prepared according to Vetter & Knappe (1971). Azotoflavin from *A. vinelandii* was prepared as described by Benemann *et al.* (1969).

RESULTS

Klebsiella nitrogenase activity

Pyruvate and formate, both key intermediates in the anaerobic metabolism of glucose by coliform bacteria (Wood, 1961), provide reducing equivalents for nitrogen fixation when supplied to crude nitrogenase extracts of *Klebsiella pneumoniae* (Table 1). The rate of nitrogenase activity supported by formate varied; in some experiments the rate was equal to that with pyruvate, while in other experiments the rates were very low. The reducing power of malate and NADPH (NADH was not tested) could also be coupled to the *Klebsiella* nitrogenase system. While the dithionite-supported activity appears to be quite low (Eady *et al.* 1972), the protein concentration used in this experiment was optimized for the pyruvate- and formate-supported and not the dithionite-supported nitrogenase activity. The high concentration of crude extract protein used in this experiment (10 mg)

Table 1. *Sources of reducing power for K. pneumoniae nitrogenase*

The reaction mixture (final volume, 1.5 ml) contained 10 mg *K. pneumoniae* extract and the following (in μ mol): HEPES buffer (pH 7.4), 50; $MgCl_2$, 5; creatine phosphate, 40; ATP, 4; creatine phosphokinase, 0.005 mg; and electron donor substrates as indicated. Gas phase, argon 73 % and acetylene 27 %; reaction time, 10 min; temperature, 30 °C.

Reductant	Ethylene formed (nmol/min)
None	3.7
Pyruvate (67 mM)	58.5
Formate (67 mM)	20.6
Malate (67 mM)	16.0
NADPH generating system*	15.3
Dithionite (13 mM)	57.0

* See Benemann *et al.* (1971) for details.

was probably too high (and consequently inhibitory) for optimum rates of dithionite-supported nitrogenase activity, since in other experiments rates ranging between 180 and 345 nmol ethylene formed/min have been observed for dithionite-supported nitrogenase activity when about half this amount of protein was used.

Dithionite-, pyruvate- and formate-supported nitrogenase activity in fresh extracts was linear for about 15 min; the same was assumed for malate- and NADPH-supported activity. The addition of creatine phosphate to the reaction mixture (anaerobically with a syringe) after 15 min brought the nitrogenase activity back to its original linear rate. This observation indicates that the fresh *Klebsiella* extracts have extremely active ATPase activities. After several days of storage of the extract at -20 °C, the nitrogenase activity was linear for at least 30 min.

Evidence which may suggest a similarity between *Klebsiella* and *E. coli* in the generation and transport of low-potential electrons is shown in Table 2 and Fig. 1. Pyruvate-supported nitrogenase activity in 4-day-old *Klebsiella* extracts is approximately one-tenth the level of that in fresh extracts. The low level of pyruvate-supported nitrogenase activity was stimulated fivefold by the addition of extract from *E. coli* grown anaerobically on glucose (Table 2). It appears that *E. coli* extract replaced the component(s) of the *Klebsiella* pyruvate 'hydrogen-donating system' that was inactivated with age, thereby restoring the pyruvate-

Table 2. *Effect of E. coli extract in coupling pyruvate-supported nitrogenase activity in a Klebsiella extract*

Reaction mixture as in Table 1 except that the *Klebsiella* nitrogenase extract (10 mg protein) was 4 days old and had low pyruvate-supported nitrogenase activity and *E. coli* extract was added as indicated. The pyruvate-supported nitrogenase activity of the freshly prepared extract (same protein concentration) was 60.8 nmol ethylene formed/min. A control showed that the dithionite-supported nitrogenase activity of this 4-day-old *Klebsiella* extract was unimpaired. Reaction time, 20 min.

<i>E. coli</i> extract added (mg)	<i>Klebsiella</i> nitrogenase activity (ethylene formed, nmol/min)
0.0	6.5
1.9	14.6
3.8	17.4
7.6	25.2
11.4	31.1

supported nitrogenase activity of the *Klebsiella* extract. No attempt was made to replace the *E. coli* extract with *K. pneumoniae* flavodoxin.

One component from *E. coli* extract that is capable of functioning in the *Klebsiella* nitrogenase system is the flavodoxin (Fig. 1). Furthermore, *E. coli* flavodoxin appeared to couple electron flow to the nitrogenase of the coliform *Klebsiella* more efficiently than to the nitrogenase of a noncoliform, *A. vinelandii*. The two nitrogenase extracts had previously been treated on a DEAE-cellulose column to remove the endogenous electron carriers, and the protein concentrations were then adjusted so that both had equal dithionite-supported nitrogenase activity. When the reducing power of illuminated chloroplasts was coupled to the respective nitrogenases by equal concentrations of *E. coli* flavodoxin, the rate of *Klebsiella* nitrogenase activity was more than double that of *Azotobacter* nitrogenase.

Isolation of *Klebsiella* electron carriers

The *Klebsiella* nitrogenase system is unlike that of *A. vinelandii*, *B. polymyxa* or *Rhizobium* root nodule bacteroids in that the reducing power of illuminated chloroplasts cannot

Fig. 1. Comparison of ability of (●) *K. pneumoniae* and (○) *A. vinelandii* nitrogenases to couple to photoreduced *E. coli* flavodoxin. Either *K. pneumoniae* or *A. vinelandii* nitrogenase, 0.1 ml, and *E. coli* flavodoxin, 0.02 μmol, were added to a reaction mixture (final volume, 1.5 ml) containing washed ferredoxin-free spinach chloroplasts (0.3 mg chlorophyll), and the following (in μmol): ascorbate, 10; 2,6-dichlorophenol indophenol (DPIP) (ascorbate-reduced DPIP replaced water as a source of electrons in the chloroplast fragments), 0.05; HEPES buffer (pH 7.4), 50; Mg^{++} , 5; creatine phosphate, 40; ATP, 4; and creatine phosphokinase, 0.005 mg. Light intensity, saturating; gas phase and temperature as in Table 1; reaction time, 20 min.

be coupled to a freshly prepared nitrogenase extract without the addition of soluble ferredoxin. Only after prolonged freezing of the *Klebsiella* extracts could electrons from illuminated chloroplasts be coupled to the nitrogenase (Table 3). Treatment of these nitrogenase extracts on a DEAE-cellulose column removed a factor that was essential for chloroplast-supported nitrogenase activity. Because the extract that passed through the DEAE-cellulose column still had high rates of dithionite-supported nitrogenase activity, it was assumed that DEAE-cellulose had removed an electron carrier component that was

Table 3. *Chloroplast-linked Klebsiella nitrogenase activity*

The reaction mixture and conditions of assay were similar to those described in Fig. 1 [with the source of reducing power for the nitrogenase reaction being washed (ferredoxin-free) spinach chloroplasts (0.3 mg chlorophyll)]. A 0.2 ml sample (approx. 6.1 mg protein) of *Klebsiella* nitrogenase extract was assayed after each successive treatment.

Treatment of extract	Ethylene formed (nmol/min)
None (freshly prepared extract)	0.7
-20 °C, 24 days	24.0
-20 °C, 38 days	33.7
DEAE-cellulose treated	6.3
DEAE-cellulose treated extract + 0.1 ml DEAE-cellulose eluate*	30.6

* The electron carrier fraction was eluted from the DEAE-cellulose column with 0.02 M-HEPES buffer, pH 7.4, containing 0.8 M-NaCl.

Fig. 2

Fig. 3

Fig. 2. Effectiveness of a *Klebsiella* DEAE-cellulose eluate in coupling electrons between illuminated chloroplasts and *Klebsiella* nitrogenase. Reaction time and mixture as in Fig. 1 with *Klebsiella* nitrogenase, 6.5 mg, and *Klebsiella* DEAE-cellulose eluate electron carrier fraction as indicated.

Fig. 3. Chromatography of *Klebsiella* electron carriers on DEAE-cellulose. Before collection of 4.5 ml fractions, the column was eluted with 150 ml phosphate buffer (pH 7.3) containing 0.1 M-NaCl. This was followed with buffer containing NaCl as indicated. The profile of fractions A and B was determined by absorbance at 450 nm, of fraction C at 630 nm, and of fraction D at 460 nm. o—o, Biologically active fractions; o---o, biologically inactive fractions.

essential in coupling electron flow between the chloroplasts and nitrogenase. Addition of this crude electron carrier fraction back to the DEAE-cellulose treated *Klebsiella* extract restored the chloroplast-supported nitrogenase activity, the degree of restoration being dependent on the concentration of the electron carrier fraction (Fig. 2).

The *Klebsiella* electron carriers were purified from a butanol extract on a DEAE-cellulose chromatography column eluted with buffer containing increasing concentrations of NaCl. The resulting elution pattern of coloured proteins (designated fractions A, B, C and D) and the salt concentrations required for their elution are shown in Fig. 3. The dashed line indicates those protein fractions with no biological activity; the solid line (fractions

Fig. 4

Fig. 5

Fig. 4. Absorption spectra of flavodoxin from *K. pneumoniae*. The flavodoxin was reduced to the semiquinone form by the addition, under anaerobic conditions, of microlitre quantities of 10 mM-sodium dithionite. Insert, the complete spectrum of oxidized *Klebsiella* flavodoxin.

Fig. 5. Effectiveness of *Klebsiella* flavodoxin in coupling the reducing power of illuminated chloroplasts to *K. pneumoniae* nitrogenase. Reaction mixture as described in Fig. 1, with 7.8 mg of *Klebsiella* nitrogenase extract and the concentration of *Klebsiella* flavodoxin as indicated.

B and D) indicates proteins with biological activity in the chloroplast–nitrogenase reaction. On several occasions fractions C and D eluted as a single band. After this chromatographic separation on DEAE-cellulose, these four fractions were concentrated and chromatographed separately on Sephadex G-50. After Sephadex G-50 chromatography, fractions A, B and C, while not homogeneous, appeared on gel electrophoresis to be free from other coloured proteins; fraction D was still a mixture of at least three different coloured proteins. Because the yield was low, purification of fraction D was not continued.

Properties and biological activity of the Klebsiella electron carriers

Fraction A was yellow and had the spectrum of a flavoprotein. It showed no electron carrier activity in the ‘chloroplast–nitrogenase’ assay or in the photoreduction of NADP^+ by illuminated chloroplasts. Its biological activity is unknown.

That fraction B was also a flavoprotein was indicated by its optical spectrum (Fig. 4). The complete spectrum of the flavoprotein in the oxidized form has absorption maxima at 277, 370 and 452 nm, and is shown in the insert to Fig. 4. On the addition of small quantities of sodium dithionite, the flavodoxin was reduced to the blue semiquinone form, a species with a broad absorbance maximum at 580 to 620 nm. Although not shown in this Figure, the addition of more dithionite reduced the blue semiquinone species to the colourless

Fig. 6

Fig. 6. Molecular weight determination of *Klebsiella* flavodoxin by gel filtration. The protein markers (5 mg each except for *C. pasteurianum* ferredoxin which was 1 mg) were applied to a Sephadex G-100 (superfine) column (1.5 × 80 cm) equilibrated and eluted with 0.02 M-phosphate buffer pH 7.4, 0.5 M-NaCl and 0.01 M-2-mercaptoethanol. The flavodoxin was applied and eluted separately. The flow rate was approx. 2.2 ml/h.

Fig. 7

Fig. 7. Effectiveness of *Klebsiella* fraction D in coupling the reducing power of illuminated chloroplasts to *K. pneumoniae* and *A. vinelandii* nitrogenases. Reaction mixture as in Fig 1, with the volume of fraction D (1.4 mg protein/ml) as indicated, and either (○) *A. vinelandii* nitrogenase extract, 7.5 mg protein, or (●) *K. pneumoniae* nitrogenase extract, 6.7 mg protein (both had previously been treated with DEAE-cellulose to remove endogenous electron carriers).

hydroquinone (fully reduced) form. Some variation from one preparation of flavodoxin to another in the 370 to 452 nm absorbance ratio was observed. Although in these preparations (Fig. 4) the 370 to 452 nm absorbance ratio was slightly greater than one, in other flavodoxin preparations this ratio was slightly less than one. There is no immediately apparent explanation for this observation.

Fraction B coupled electron transfer between illuminated chloroplasts and *Klebsiella* nitrogenase (Fig. 5). The finding that this flavoprotein functions in the nitrogenase reaction suggests that fraction B is a *Klebsiella* flavodoxin, as only flavodoxins among the flavoproteins are known to reduce nitrogenase. There was not a sufficient amount of pure *Klebsiella* flavodoxin available to determine its extinction coefficient successfully; therefore, the extinction coefficient used to determine the flavodoxin concentration was that of free FMN ($12.1 \text{ mm}^{-1} \text{ cm}^{-1}$ at 450 nm). Using this value probably underestimates the concentration of flavodoxin as the extinction coefficient of the other flavodoxins varies from about 8.5 to $10.5 \text{ mm}^{-1} \text{ cm}^{-1}$ at 450 nm (Hinkson & Bulen, 1967; Mayhew & Massey, 1969; Vetter & Knappe, 1971; Cusanovich & Edmondson, 1971; Mayhew, 1971). Thus, while the flavodoxin concentrations shown in Fig. 5 may be low (10 to 15%), the point to be emphasized here is that this *Klebsiella* flavoprotein does in fact couple to the nitrogenase and is effective at relatively low concentrations.

The molecular weight of *Klebsiella* flavodoxin was estimated to be 21000 ± 1000 by the gel filtration method (Andrews, 1965) on Sephadex G-100 (superfine grade) using *Clostridium*

Table 4. *The synergistic effect of methyl viologen on azotoflavin- and Klebsiella flavodoxin-coupled nitrogenase activity*

The reaction mixture and conditions of assay were similar to those described for Fig. 1 with DEAE-treated *Azotobacter* extract (5.9 mg protein) as the source of nitrogenase, illuminated chloroplasts (0.28 mg chlorophyll) as the source of reductant, and electron carriers as indicated.

Addition	Ethylene formed (nmol/min)
Azotoflavin (21.5 μM)	15.8
Methyl viologen (0.67 μM)	14.5*
Azotoflavin (21.5 μM) + methyl viologen (0.67 μM)	78.0
Klebsiella flavodoxin (7.1 μM)	3.0
Methyl viologen (0.67 μM)	12.1
Klebsiella flavodoxin (7.1 μM) + methyl viologen (0.67 μM)	62.0
None	0

* The rate of nitrogenase activity one observes with low concentrations of methyl viologen probably depends on how completely the endogenous electron carriers are removed from the *Azotobacter* extract (cf. Table 2; Yoch, 1972).

pasteurianum ferredoxin, cytochrome *c*, myoglobin, chymotrypsinogen and ovalbumin as standards (Fig. 6).

Klebsiella flavodoxin resembles azotoflavin (Hinkson & Bulen, 1967; Benemann *et al.* 1969) in that it does not mediate the photochemical reduction of NADP^+ by ferredoxin-free chloroplast fragments at low concentrations under aerobic conditions. Klebsiella flavodoxin further resembles azotoflavin (Yoch, 1972) in that catalytic concentrations of methyl viologen greatly increase its effectiveness as a link between the reducing power generated by spinach chloroplasts and DEAE-cellulose-treated *Azotobacter* nitrogenase (Table 4). The stimulatory effect of methyl viologen on azotoflavin- and Klebsiella flavodoxin-mediated nitrogenase activity may be a phenomenon common to all flavodoxins.

Fraction C was a blue protein that showed a broad absorption peak with a maximum at 630 nm. Although this protein spectrally resembles the copper-containing *Pseudomonas* blue protein (azurin) isolated by Horio (1958*a, b*), the Klebsiella blue protein differs in that it could not be reduced by ascorbate but was readily reduced to a colourless form by sodium dithionite. The fact that the Klebsiella blue protein was not reduced by ascorbate appears to make it unlike any of the known blue copper-containing proteins (see review by Malkin, 1973). The biological function of the Klebsiella blue protein is unknown.

Fraction D was brown in colour. Disc gel electrophoresis showed this fraction to be a combination of several different coloured proteins and therefore it had no distinct spectral characteristics. Klebsiella fraction D coupled the reducing power of illuminated chloroplasts equally well to the nitrogenases of *Klebsiella* and *Azotobacter* (Fig. 7). The low level of Klebsiella nitrogenase activity in the absence of added fraction D indicates that a small amount of endogenous electron carrier contaminated the Klebsiella nitrogenase extract. Like Klebsiella flavodoxin, fraction D did not function as a substitute for plant ferredoxin in the photoreduction of NADP^+ by chloroplast or blue-green algae fragments. The identity of the active electron transport component in fraction D is unknown.

DISCUSSION

The restoration of pyruvate-supported nitrogenase activity in *Klebsiella* extracts by an extract prepared from glucose-fermenting *E. coli* cells and the greater efficiency of photo-reduced *E. coli* flavodoxin in coupling to *Klebsiella* nitrogenase than to *Azotobacter* nitrogenase suggest that *K. pneumoniae* and *E. coli* may generate and transport electrons of low oxidation-reduction potential by a similar mechanism.

The predominant electron carrier in *Klebsiella*, as in *E. coli* (Vetter & Knappe, 1971) appears to be the flavodoxin. However, unlike *E. coli* flavodoxin (Vetter & Knappe, 1971), the one from *Klebsiella* does not substitute for plant ferredoxin in the reduction of NADP^+ by illuminated chloroplasts. In this respect *Klebsiella* flavodoxin resembles a flavoprotein electron carrier from *A. vinelandii* called azotoflavin or, more recently, *Azotobacter* flavodoxin. Although van Lin & Bothe (1972) reported that azotoflavin functions in this chloroplast reaction when high concentrations (100 to 200 μM) of the flavoprotein are used under strictly anaerobic conditions, I was unable to verify this claim. Similarly negative results were obtained with *Klebsiella* flavodoxin under these conditions.

The identity of the second electron carrier in *Klebsiella*, designated in this report as fraction D, is not known; by analogy with *E. coli*, one might expect the active component to be a coliform ferredoxin. Whereas no biological activity was reported for *E. coli* ferredoxin (Vetter & Knappe, 1971), *Klebsiella* fraction D does have one ferredoxin-like activity in that it reduces nitrogenase.

The significance of finding two electron carriers in *Klebsiella*, each capable of coupling reducing power to *Klebsiella* nitrogenase, is not understood at the present time. It may be that the pyruvate- and formate-coupled nitrogenase systems function specifically with one or another of these carriers. In *Azotobacter* (Benemann *et al.* 1971) and *Rhizobium* root nodule bacterioids (Wong, Evans, Klucas & Russell, 1971), both a ferredoxin and a flavodoxin-like protein are required to couple electron transport between NADPH and the nitrogenase.

In one respect the *Klebsiella* nitrogenase system resembles that of the photosynthetic bacterium *Chromatium*, in that reducing power from illuminated chloroplasts does not couple to the nitrogenase unless exogenous ferredoxin is added to the reaction mixture (Yoch & Arnon, 1970). In *Chromatium* the ferredoxin appears to be bound to the membranes, as detergent (Triton X-100) treatment greatly increases the yield of ferredoxin isolated from these cells (K. T. Shanmugam, personal communication). In untreated *Chromatium* extracts, electrons from illuminated chloroplasts presumably do not couple to the nitrogenase because the ferredoxin is inaccessibly bound to the bacterial photosynthetic membranes. In *Klebsiella*, the reducing power from chloroplasts coupled to the nitrogenase only after a prolonged freezing of the extract (see Table 4), suggesting that this process might be releasing electron carriers that were bound or complexed in such a way as to be previously inaccessible to the electrons from chloroplasts. Although the evidence is preliminary, it would appear that the electron carriers that couple to the nitrogenase of *Klebsiella* and *Chromatium* are not soluble, as they are in other nitrogen-fixing microorganisms.

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