

## Immunochemical Analysis of Respiratory-Chain Components of *Micrococcus luteus* (*lysodeikticus*)

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Membrane-bound antigens of the respiratory chain of *Micrococcus luteus* were analyzed by crossed immunoelectrophoresis after growth of the organism in the presence of  $^{59}\text{Fe}$ , the flavin adenine dinucleotide-flavin mononucleotide precursor D-[2- $^{14}\text{C}$ ]riboflavin, or the heme precursor 5-amino-[4- $^{14}\text{C}$ ]levulinic acid. Using zymograms and procedures of selective extraction in conjunction with autoradiography, it was possible to resolve and partially characterize a number of antigens. Succinate dehydrogenase (EC 1.3.99.1) was shown to possess covalently bound flavin and nonheme iron and was possibly present as a complex with cytochrome. Three other dehydrogenases, namely, NADH dehydrogenase, NAD(P)H dehydrogenase (EC 1.6.99.3), and malate dehydrogenase (EC 1.1.1.37), contained flavin in noncovalent linkage, the NAD(P)H dehydrogenase also possessing nonheme iron. Four other discrete antigens (or antigen complexes) containing both iron and heme centers also resolved, as were two minor immunogens possessing iron as the sole detectable prosthetic group.

The respiratory chain of the gram-positive, aerobic bacterium *Micrococcus luteus* (*lysodeikticus*) is thought to consist of the following sequence of redox carriers (6, 15, 26):

NADH  
dehydrogenase  
  
malate  
dehydrogenase  
  
lactate  
dehydrogenase  
  
succinate  
dehydrogenase

cyt.  $b_{556}$  → MQ9 → cyt.  $b_{560}$  → (NHI) → cyt.  $c_{550}$  → cyt.  $a_{601}$  →  $\text{O}_2$

where MQ9 and NHI represent menaquinone-9 and nonheme iron protein, respectively. Attempts to resolve and study the properties of individual respiratory-chain components by the classical biochemical approach of purification followed by characterization has met with only limited success. Best characterized of these components is NADH dehydrogenase (EC 1.6.99.3), which has been shown to be a flavo-protein of subunit molecular weight about 70,000 (10, 30, 31). Succinate dehydrogenase (EC 1.3.99.1) and cytochrome  $b_{556}$  have been partially purified (12, 14), and complexes of NADH dehydrogenase · cytochrome  $b_{556}$ , malate dehydrogenase · cytochrome  $b_{556}$  and cytochrome  $b_{560}$  · cytochrome  $c_{550}$  · cytochrome  $a_{601}$  have also been described (6, 15, 28).

Several years ago, the high-resolution two-dimensional technique of crossed immunoelectrophoresis (CIE) was applied to the study of *M. luteus* membranes, and the results, in terms of an analysis of respiratory-chain components, were encouraging (18, 21, 23). Twenty-seven membrane immunogens were resolved by this

technique, among them antigens possessing NADH dehydrogenase (two separate components), succinate dehydrogenase, and malate dehydrogenase (EC 1.1.1.37) activities. All four enzyme antigens were expressed solely on the cytoplasmic face of the membrane as judged from the results of progressive immunoadsorption experiments conducted with protoplasts and isolated membranes (21, 23).

In this communication we extend the immunochemical study of the *M. luteus* respiratory chain and, by analysis of membranes derived from cells grown in the presence of  $^{59}\text{Fe}$ , the flavin adenine dinucleotide and flavin mononucleotide precursor D-[2- $^{14}\text{C}$ ]riboflavin, or the heme precursor 5-amino-[4- $^{14}\text{C}$ ]levulinic acid, identify membrane antigens possessing iron centers, bound flavin, and heme groups.

A preliminary account of this work has been presented (B. A. Crowe and P. Owen, Soc. Gen. Microbiol. Q. 7:72, 1980).

## MATERIALS AND METHODS

**Growth of cells and preparation of plasma membranes.** Cells of *M. luteus* (NCTC 2665) were grown in either peptone-water-yeast extract medium (25) or defined medium (22) supplemented when necessary with the appropriate radioisotope. To achieve iron-labeled cells, the growth medium was supplemented with  $^{59}\text{FeCl}_3$  (1.2 to 1.3  $\mu\text{Ci/ml}$ ). The concentration of (unlabeled) iron in the defined medium was reduced by a factor of 10 (i.e., to 0.4  $\mu\text{g/ml}$ ) to improve incorporation of the label. In the case of riboflavin labeling, the growth medium was supplemented with D-[2- $^{14}\text{C}$ ]riboflavin (3.0  $\mu\text{Ci/ml}$ ) and was protected from the light at all times to prevent photodecomposition of the label. To achieve labeling of the heme groups, cells were first grown in the presence of unlabeled 5-amino-levulinic acid (final concentration, 2  $\mu\text{g/ml}$ ) and then inoculated into media containing 5-amino-[4- $^{14}\text{C}$ ]levulinic acid (0.65  $\mu\text{Ci/ml}$ ). Plasma membranes were prepared from osmotically lysed cells (22, 25) and were routinely washed four times in 50 mM Tris-hydrochloride buffer (pH 7.5) before storage at  $-70^\circ\text{C}$ .

**Preparation of Triton X-100 extracts of membranes.** Plasma membranes (final protein concentration, 18 to 22 mg/ml) were extracted twice with 4% Triton X-100 in 50 mM Tris-hydrochloride buffer (pH 7.5) as previously described (21). Insoluble material was removed by centrifugation at 30,000  $\times g$  for 45 min at  $20^\circ\text{C}$ .

**CIE and related immunological techniques.** Antiserum to plasma membranes was raised in rabbits by procedures outlined by Owen and Salton (21). Sera from four rabbits were pooled (total volume, 500 ml), and the immunoglobulin fraction was purified and concentrated to 0.1 serum volume as previously described. Two such serum pools were used in the course of the experimentation.

Methods for performing CIE in the presence of Triton X-100 and for washing, drying, and staining immunoplates with Coomassie brilliant blue have been detailed elsewhere (21, 23). Immunoprecipitates possessing succinate dehydrogenase, NADH dehydrogenase, NADPH dehydrogenase (EC 1.6.99.1), malate dehydrogenase, and ATPase (EC 3.6.1.3) activities were detected by published procedures for enzyme staining (17). Development of the malate dehydrogenase zymograms could be improved by substituting NADP (final concentration, 0.2 mg/ml) for NAD in the reaction mixture. Immunoprecipitates possessing bound iron, flavin, or heme were detected by autoradiography of relevant unstained immunoplates. Selective extraction of nonheme iron and extraction of total iron was performed on wet saline-washed immunoplates as described by Owen et al. (20). Differentiation between noncovalently bound and covalently bound flavin was based on methods described by Koziol (11) and Chappelle and Picciolo (2). This involved incubating pressed immunoplates individually for 15 min at  $4^\circ\text{C}$  in 40 ml of either 10% (wt/vol) trichloroacetic acid (11) or 1 M perchloric acid (2). Both procedures result in denaturation of flavoantigens and consequent labilization of flavin prosthetic groups not in covalent linkage to protein. The acid-extracted immunoplates were then rinsed six times in a large excess of distilled water before pressing and air drying. Immunoplates treated with appropriate volumes of distilled water

were run as controls. The length of time from initial extraction to air drying did not exceed 45 min.

**Characterization of noncovalently bound flavin.** The nature of the noncovalently bound label associated with membranes obtained from cells grown in the presence of D-[2- $^{14}\text{C}$ ]riboflavin was assessed by paper chromatography of a water-soluble extract obtained after heat denaturation of the labeled membranes. Riboflavin-labeled membranes (580  $\mu\text{g}$  of protein) were heated to  $80^\circ\text{C}$  for 10 min, and the denatured protein was pelleted by centrifugation at 10,000  $\times g$  for 5 min in a Beckman microfuge. The supernatant fraction (50  $\mu\text{l}$ ) containing released radiolabel was spotted onto Whatman no. 3 paper, and ascending chromatography was performed with the upper phase of an *n*-butanol-acetic acid-water mixture (4:1:5, by volume) as solvent. Riboflavin, flavin mononucleotide, and flavin adenine dinucleotide were used as standards at loadings of 0.5  $\mu\text{g}$ . Flavins were located by their fluorescence in UV light, and these were compared with the positions of the radiolabeled components detected by autoradiography.

**Analytical methods and chemicals.** Protein was determined by a modification of the method of Lowry et al. (13) that eliminates interference by Triton X-100 (5). Bovine serum albumin was used as the standard.

$^{59}\text{FeCl}_3$  (9.6 mCi/mg of iron), D-[2- $^{14}\text{C}$ ]riboflavin (60.6 mCi/mmol), and 5-amino-[4- $^{14}\text{C}$ ]levulinic acid hydrochloride (58 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, U.K.

Triton X-100 used for extraction of membranes was purchased from Research Products International (Elk Grove, Ill.), and agarose ( $-M_r > 0.2$ ) was from Miles Chemical Co. (Stoke Poges, U.K.)

## RESULTS

**General comments regarding identification of immunoprecipitates.** For reasons probably relating to the use of different serum pools and to differing conditions of cell culture, the CIE immunoprecipitate profiles obtained for  $^{59}\text{Fe}$ -, [ $^{14}\text{C}$ ]riboflavin-, and [ $^{14}\text{C}$ ]aminolevulinate-labeled membranes did not bear an immediate resemblance to each other or to the standard reference profile of 27 immunoprecipitates published by Owen and Salton (23) for membranes of *M. luteus*. In view of the obvious problem in identification, strict criteria were used in ascribing numbers to immunoprecipitates. An immunoprecipitate was only assigned a number in the published reference profile of Owen and Salton (23) if the component antigen could be unambiguously identified by virtue of some unique property, e.g., enzymatic activity. Thus, the identities of succinate dehydrogenase (no. 8), NADH dehydrogenase (no. 10 and 13), and malate dehydrogenase (no. 21) were established for each preparation by zymograms. Differentiation between the two NADH dehydrogenases was made on their differential ability to reduce tetrazolium red (24). Immunogens which did not fall into this category but which, from a close study of relevant immunoplates, showed great similar-

ity in electrophoretic mobilities and characteristics of immunoprecipitation to components of the standard reference profile (23) were ascribed the appropriate numbers and asterisked to indicate the tentative nature of the identification (e.g., antigens 23\* and 26\*). New antigens which had no obvious counterparts in the standard reference profile were given numbers over 27 (no. 28 to 32). The problem of correlating relationships between immunoprecipitates in the CIE profiles of the three types of radiolabeled membrane preparations was circumvented in large part either by conducting co-CIE or tandem-CIE experiments (or both) with the relevant preparations or by performing zymograms. Difficulties were encountered, however, in the cases of immunogens 29 and 31. These antigens generated immunoprecipitates which could be visualized by autoradiography but less readily by protein staining. Reactions of identity or nonidentity were thus difficult to establish in protein-stained profiles.

The results presented below are based on a study of over 150 immunoplates run under conditions of different antigen and antibody concentrations.

**CIE analysis of  $^{59}\text{Fe}$ -labeled membranes.** Typical CIE profiles obtained for first Triton X-100 extracts of  $^{59}\text{Fe}$ -labeled *M. luteus* membranes are shown in Fig. 1A and B. Autoradiography of these and other gels revealed that at least nine discrete immunoprecipitates (no. 8, 13, 23\*, 26\*, and 28 to 32) possessed bound iron (Fig. 1C and D and 2A). The major iron-containing immunogen (no. 8) was identified as succinate dehydrogenase, and another (no. 13) could be shown to be an NADH dehydrogenase which could also oxidize NADPH (Fig. 1E and F). Two other iron-containing antigens (no. 29 and 30) were only observed in membranes obtained from cells grown in defined media (Fig. 1B and D), and another (no. 32) was only observed in second Triton X-100 extracts (not shown) and was not studied further. In addition, parts of immunoprecipitates 11 and 21 were occasionally observed to be labeled (see Fig. 1D). However, for reasons similar to those detailed below, it seems probable that the labeling in these instances is largely a consequence of antigen entrapment (1, 24). None of the iron-containing immunogens originated from the cytoplasm as judged by CIE analysis of  $^{59}\text{Fe}$ -labeled membranes and cyto-

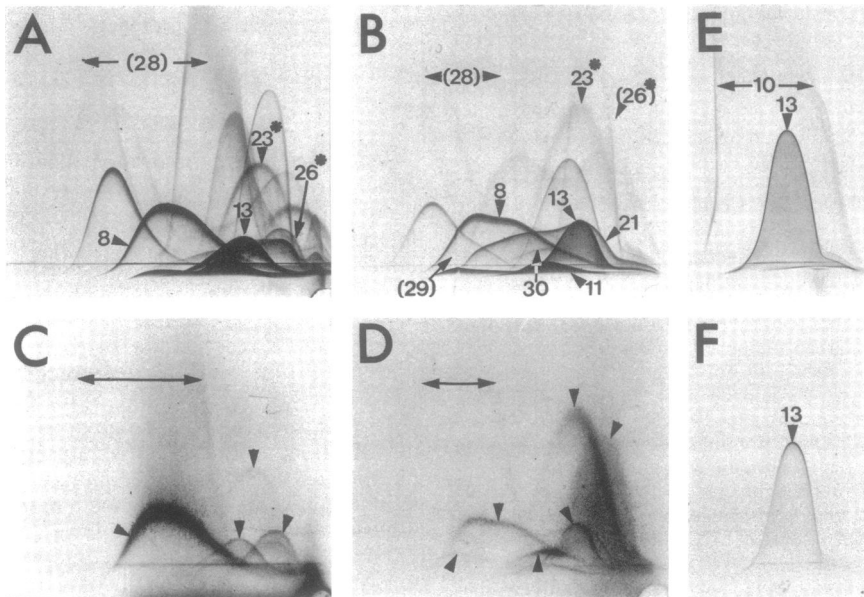


FIG. 1. CIE analysis of  $^{59}\text{Fe}$ -labeled membranes. First Triton X-100 extracts of  $^{59}\text{Fe}$ -labeled membranes of *M. luteus* which had been grown in peptone-water-yeast extract medium (A, E, and F) or in defined medium (B) were analyzed against antimembrane serum (4.5 mg of protein per ml of gel). The amounts of membrane protein analyzed were 46 (A), 42 (B), and 90 (E and F)  $\mu\text{g}$ . (A) and (B) have been stained for protein, and their corresponding 6- and 7-day autoradiograms are shown in (C) and (D), respectively. (E) and (F) have been stained for NADH and NAD(P)H dehydrogenase activities, respectively. The salient radiolabeled immunoprecipitates in (C) and (D) are arrowed, and their identities in the full spectrum of immunoprecipitates are indicated by no. 23 in (A) and (B). Parentheses indicate that the immunoprecipitate in question is not readily observed in the protein-stained profile. Due to antigen excess, immunogen 26\* has not produced a well-defined immunoprecipitate in (B). Anode is to the left and top of all gels.

plasm against both antimembrane and anticytoplasm sera.

Attempts to determine by selective extractions (20) whether the iron in individual immunoprecipitates was of the heme or nonheme form were consistently complicated by the observations that (i) treatment of CIE immunoplates with reagents designated to extract total (i.e., nonheme plus heme) iron (20) resulted in incomplete removal of the isotope (Fig. 2D) and (ii) treatment of control immunoplates with distilled water resulted in significant loss of label (Fig. 2A and B). It is unclear whether this latter observation reflects the presence of extremely labile iron-sulfur centers or of nonspecifically bound isotope. (Certainly, there was no change in the intensity of protein staining after extraction.) Despite these complications the following tentative conclusions could be drawn. Succinate dehydrogenase (antigen 8) appeared to contain the metal in the form of nonheme iron primarily, as judged from autoradiograms of immunoplates subjected to treatment with reagents that remove nonheme iron or total iron (Fig. 2). In contrast, antigens 26\*, 28, and 31 apparently possess appreciable amounts of iron in the form of the heme derivative. These results are summarized in Table 1.

**CIE analysis of flavin-labeled membranes.** Flavoprotein antigens were selectively resolved by autoradiography of CIE plates in which membranes from cells grown in the presence of D-[2-<sup>14</sup>C]riboflavin had been analyzed against antimembrane serum (Fig. 3). In this way four of the

membrane immunogens could be shown to possess flavin prosthetic groups (cf. Fig. 3A and C). These were identified by zymograms as succinate dehydrogenase (no. 8), both NADH dehydrogenases (no. 10 and 13), and malate dehydrogenase (no. 21; see Fig. 3B). Only succinate dehydrogenase (no. 8) possessed flavin in a covalently bound form as judged from autoradiograms of CIE plates which had been treated with acidic solutions known to labilize the noncovalently bound prosthetic group (Fig. 3D). Subsequent staining of the immunoplates with Coomassie brilliant blue confirmed that the reduction in peak intensity observed in autoradiograms for antigens 10, 13, and 21 after acid extraction was due solely to labilization of the isotope and not to dissociation of the antigen-antibody complexes. Moreover, chromatographic analysis of the noncovalently bound isotope present in flavin-labeled membranes (see Materials and Methods) revealed the presence of radioactive material with retentions similar to those of flavin adenine dinucleotide and flavin mononucleotide. These two components accounted for 48 and 7%, respectively, of the total noncovalently bound isotope. A third fluorescent component of unknown identity accounted for a further 34% of the radioactivity.

**CIE analysis of levulinate-labeled membranes.** CIE analysis of membranes prepared from cells grown in the presence of the heme-siroheme precursor 5-amino-[4-<sup>14</sup>C]levulinic acid revealed a number of radiolabeled immunoprecipitates upon autoradiography (Fig. 4A). Four im-

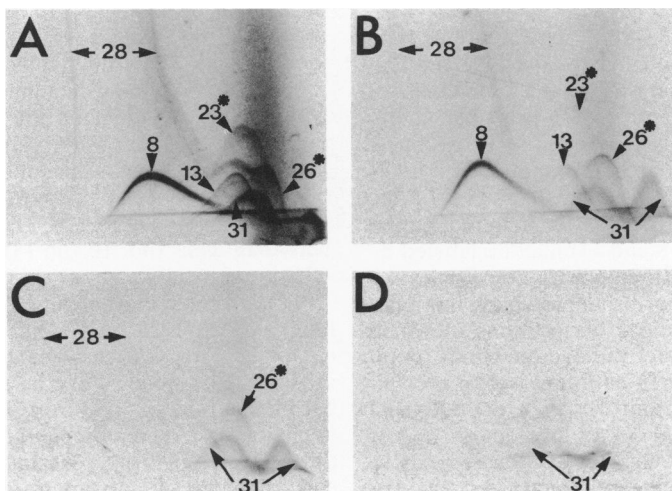


FIG. 2. Extraction of <sup>59</sup>Fe from radiolabeled immunoprecipitates. A Triton X-100 extract (45 μg of protein) of <sup>59</sup>Fe-labeled *M. luteus* membranes was analyzed by CIE against antimembrane serum (4.5 mg of protein per ml of gel) in all instances. Saline-washed immunoplates were simply dried (A), washed in distilled water (B), extracted for nonheme iron (C), or extracted for total iron (D) as detailed by Owen et al. (20). Seven-day autoradiograms of all four gels are presented. The anode is to the left and top of all gels.

TABLE 1. Prosthetic groups associated with membrane-bound immunogens of *M. luteus*

Antigen no.	Identity	Associated prosthetic group <sup>d</sup>		
		Iron <sup>b</sup>	Flavin <sup>c</sup>	Heme
8	Succinate dehydrogenase	+	+ (c)	? <sup>d</sup>
10	NADH dehydrogenase	-	+ (nc)	-
13	NAD(P)H dehydrogenase	+	+ (nc)	-
21	Malate dehydrogenase	? <sup>d</sup>	+ (nc)	-
23*	?	+	-	+
26*	?	+ (h)	-	+
28	?	+ (h)	-	+
29 <sup>e</sup>	?	+	-	-
30 <sup>e</sup>	?	+	-	-
31 <sup>e,f</sup>	?	+ (h)	-	+
32 <sup>g</sup>	?	+	ND	ND

<sup>a</sup> Possession or absence of prosthetic groups by antigens indicated by + and -, respectively.

<sup>b</sup> The presence of iron with the extraction characteristics of the heme (h) derivative is indicated in parentheses.

<sup>c</sup> The covalent (c) or noncovalent (nc) nature of the linkage is indicated in parentheses.

<sup>d</sup> Antigen entrapment makes assessment difficult.

<sup>e</sup> This antigen generates a CIE immunoprecipitate which is barely visible or difficult to discern in protein-stained profiles. Thus, one cannot rule out the possibility that the absence of a band in autoradiograms implies that the antigen is absent from the preparation, rather than that the radiolabeled prosthetic group is absent from the antigen.

<sup>f</sup> Since tests of identity could not be performed, correlation between the <sup>59</sup>Fe-labeled and levulinate-labeled species must be considered tentative.

<sup>g</sup> Only detected in second Triton X-100 extracts. ND, Not determined.

munogens (no. 23\*, 26\*, 28, and 31) gave precipitates which labeled consistently and uniformly in all preparations and at all loadings of antigen tested. It seems highly probable, therefore, that these antigens possess heme residues. Several other radioactive immunoprecipitates were also detected (Fig. 4A). However, there are indications that these latter immunoprecipitates (including no. 8 and 11) may be labeled by virtue of antigen entrapment (1, 24). For example, they are all major protein-staining immunoprecipitates, the radioactive portions of which are subtended by other radioactive bands (notably no. 26\* and 31). In addition, unlike precipitates 23\*, 26\*, 28, and 31, they do not label consistently from gel to gel. Moreover, one of the immunoprecipitates (no. 11) corresponds to ATPase (21, 23), a well-characterized enzyme which has not been reported to be associated with cytochromes (4). On balance, it seems likely that these immunoprecipitates owe their detection on autoradiograms largely to the entrapment within their antigen-antibody matrices

of antigens 26\* and 31. It should be noted, however, that antigen 8 (succinate dehydrogenase; Fig. 4B) does label faintly outside the entrapment zone. Thus, an association of this enzyme with cytochromes cannot be ruled out.

## DISCUSSION

It is evident from the data presented above and compiled in Table 1 that CIE is a suitable method for the resolution and analysis of respiratory-chain components. The relatively mild procedures involved in CIE allow retention by individual antigens of characteristic prosthetic groups, such as iron-sulfur centers and noncovalently bound flavin and heme, which are normally lost during analysis under denaturing conditions. By taking advantage of this property and using appropriately labeled membranes, it has been possible to reveal a number of novel features for the plasma membrane of *M. luteus*. Thus, the succinate dehydrogenase of this organism appears to be similar to the succinate dehydrogenases from mitochondrial sources and from *Rhodospirillum rubrum*, *Bacillus subtilis*, and *Escherichia coli* (2a, 7, 9, 20) in its possession of nonheme iron and covalently bound flavin. In addition, the NAD(P)H dehydrogenase (antigen 13) is clearly a metalloflavoprotein possessing iron, whereas antigen 10 (NADH dehydrogenase) and malate dehydrogenase appear to be simple flavoproteins. The presence in *M. luteus* membranes of two NADH dehydrogenase antigens, one of which possesses nonheme iron and can oxidize NADPH, is strikingly similar to the situation that exists in the membranes of *E. coli* ML308-225. In this gram-negative organism the iron-free flavoantigen has been identified as dihydrolipoyl dehydrogenase (EC 1.6.4.3), an enzyme which manifests diaphorase activity (19). Whether or not a similar situation occurs for *M. luteus* will require further investigation, as will an assessment of the relationship between the NADH dehydrogenase characterized by Zhukova et al. (30, 31) and two enzyme-active antigens (no. 10 and 13) demonstrated by CIE in this study.

Of the remaining antigens listed in Table 1, four (no. 23\*, 26\*, 28, and 31) are obvious contenders for cytochromes (or complexes of cytochromes) since all can be shown to possess both heme residues and iron. Judged from their intensities of immunoprecipitation, two of these (no. 23\* and 26\*) are reasonably immunogenic and would be suited to more detailed immunochemical analysis, e.g., by procedures involving precipitate excision and analysis (16; P. Owen, in O. J. Bjerrum, ed., *Electroimmunochemical Analysis of Membrane Proteins*, in press). In contrast, antigens 29 and 30 are obviously weak

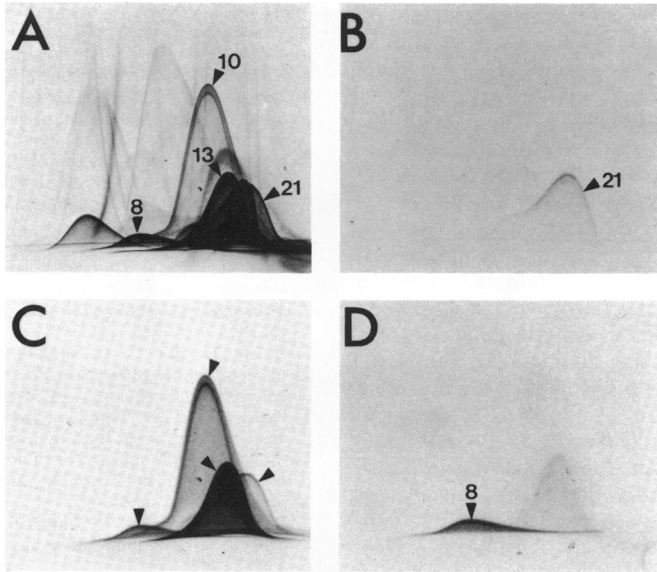


FIG. 3. CIE analysis of D-[2-<sup>14</sup>C]riboflavin-labeled membranes. Triton X-100 extracts (62 μg of protein) of riboflavin-labeled *M. luteus* membranes were analyzed against antimembrane serum (3.6 mg of protein per ml of gel), and immunoplates were stained for protein (A) or malate dehydrogenase activity (B). Identical gels were either treated with distilled water (C) or extracted for noncovalently bound flavin (D) as detailed in the text. Nine-day autoradiograms of these gels are shown in (C) and (D). The identities of the four flavoantigens are indicated by numbers in the protein-stained profile (A). The anode is to the left and top of all gels.

immunogens and appear to possess iron but not flavin or heme. These may correspond to novel oxidoreductase enzymes, ferredoxin-type carriers, or nonheme iron proteins (see above).

Several lines of evidence have suggested that some immunoprecipitates resolved by CIE for detergent-solubilized membranes may represent complexes of membrane components rather than single antigens. Such complexes, it is proposed, may represent an association of membrane components which resist dissociation and which perform some coupled biological function (reviewed in references 17 and 24). Although the results of our present investigation do not rule

out the presence of such complexes in the CIE profile of *M. luteus* membranes, neither do they provide direct evidence for their existence. Certainly, there is no precedent for thinking that the dehydrogenase antigens could be complexes of flavin-free enzymes and flavoproteins (7, 9). That no heme-containing immunogens (i.e., no. 23\*, 26\*, 28, and 31) are associated with flavoproteins, together with the observation that none of the flavoenzymes appear to coprecipitate during CIE with cytochromes (Table 1), does not favor the presence of complexes either. However, it is feasible that some of the heme-containing antigens (e.g., no. 23\*, 26\*, and 28)

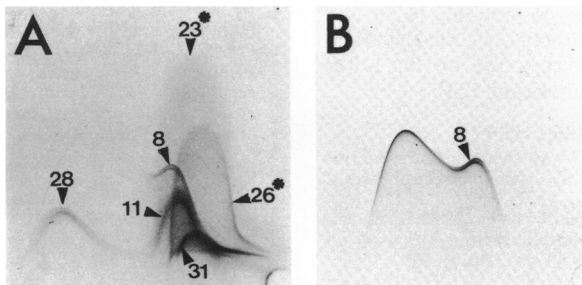


FIG. 4. CIE analysis of 5-amino-[4-<sup>14</sup>C]levulinic acid-labeled membranes. Triton X-100 extracts (86 μg of protein) of levulinate-labeled membranes were analyzed against antimembrane serum (3.6 mg of protein per ml of gel). A 15-day autoradiogram of such a gel is shown in (A), and a similar gel stained for succinate dehydrogenase activity is presented in (B). All radiolabeled immunoprecipitates are identified by numbers in (A). The anode is to the left and top of both gels.

may represent complexes of cytochrome(s) and nonheme iron proteins. Such an interpretation is not at variance with the results of experiments involving iron extraction.

A persistent problem which complicates this type of analysis is that of antigen entrapment (1, 24). The situation for the succinate dehydrogenase antigen (no. 8) clearly illustrates this point. The heme label detected in association with the cathodal leg of the corresponding immunoprecipitate could very well have originated by virtue of antigen entrapment. However, it has been reported that succinate dehydrogenases from this and other organisms retain an intimate association with type *b* cytochromes in the presence of nonionic detergents (1a, 8, 29). Furthermore, low-temperature spectroscopic analysis of material precipitated from Triton X-100-solubilized membranes of *M. luteus* by serum specific for succinate dehydrogenase has demonstrated conclusively the presence of cytochrome *b*<sub>556</sub> (1a). Thus, the heme label detected for antigen 8 (Fig. 4) could reflect, in part, an association with cytochrome. That the label extends outside the entrapment zone would support this interpretation.

Irrespective of this point, it is clear that CIE offers an excellent opportunity to analyze in detail important components of the bacterial respiratory chain. For example, established procedures of precipitate excision combined with either polypeptide analysis on sodium dodecyl sulfate-polyacrylamide gels (16; Owen, in press) or immunization (3) are presently being used to establish the polypeptide composition of key antigens and to generate to them specific sera. Similarly, progressive immunoadsorption experiments (17; Owen, in press) performed with protoplasts and isolated membranes will allow a direct assessment of the disposition of cytochromes and nonheme iron proteins in the membrane (27). In this respect, it is pertinent to note that experiments of this latter type have already demonstrated that antigen 23 of *M. luteus* membranes (which we think is analogous to the heme-containing ferroantigen 23\* described in this communication) is expressed on the outer surface of the plasma membrane (23).

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