Plasmid-determined Transformation of *cis*-Abienol and Sclareol in *Rhodococcus erythropolis* JTS-131

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cis-Abienol-transformable bacterium JTS-131 was identified as *Rhodococcus erythropolis*. This bacterium had a plasmid, pCA4. Cured strains which had no plasmid were obtained by mitomycin C treatment. These cured strains lost the ability to oxidize the C-18 methyl of *cis*-abienol and sclareol. From these results, it was considered that C-18 methyl oxidation of these labdanes was determined by the plasmid, pCA4, in this bacterium.

In the previous paper,¹⁾ we reported the microbial transformation of *cis*-abienol [(12*Z*)-labda-12,14-diene] and sclareol [labd-14-en-13 β -ol] by the soil bacterium, JTS-131, together with their transformation pathways.

In other papers,^{2~4)} we reported that *Nocardia restricta* JTS-162 transformed *cis*-abienol and sclareol. And, it was considered that C-18 methyl oxidation and A-ring splitting of these labdanes were determined by the plasmid, pCA1, in this strain.

The present report describes taxonomic studies on bacterium JTS-131 and evidence which indicates that the transformation pathways of *cis*-abienol and sclareol in bacterium JTS-131 may be determined by a plasmid.

MATERIALS AND METHODS

Microorganisms. Bacterium JTS-131 isolated as a *cis*-abienol-transformable microorganism¹⁾ was used in this study. *Rhodococcus erythropolis* ATCC 4277 was used as the type culture.

Morphological characteristics. Cell form and growth on L-agar, nutrient agar, and rich and poor agars⁵⁾ were observed after 1 to 7 days incubation. Huckers modification method⁶⁾ was employed for gram-staining. Motility was investigated by the hanging drop method, and flagellation was confirmed by the staining method of Toda.⁷⁾ The acid-fast test was carried out by the method of Ziehl-Neelsen.⁸⁾ For the milk culture, B.P.C. was used as a pH indicator.

Biochemical characteristics. Production of acid from carbohydrates was observed for 2 weeks at 28°C, with a medium composed of 3.0g of pepton, 2.5g NaCl, 5.0g carbohydrate and 1000 ml distilled water, adjusted to pH 7.2 and colored with B.C.P.

Assimilation of organic compounds as sources of carbon and energy for growth was examined by the method of Stanier *et al.*⁹⁾ In general, a concentration of 1.0% was used except for sugars and aromatic compounds which were used at concentrations of 0.2% and 0.025%, respectively. Nitrite formation from nitrate was examined in nitrate and succinate-nitrate broth¹⁰⁾ after 1, 3, 5 and 8 days incubation at 28°C. Extracellular activity was determined after 1, 3 and 7 days incubation at 28°C with a DNase test medium (Eiken Chemical Co., Ltd. Japan). Urease was examined by the method of Gordon *et al.*¹¹⁾

Isolation of DNA and determination of GC content. Bacterium JTS-131 was cultured in L-broth containing glycine at a final concentration of 1%.¹²⁾ Isolation of DNA was carried out by Marmur's method.¹³⁾ The DNA base composition was calculated from its thermal denaturation temperature (T_m).

Determination of meso-diaminopimic acid (meso-DAP). Bacterium JTS-131 was cultured in L-broth for 3 days at 28°C with shaking. Preparation and hydrolysis of cell walls was carried out by the method of Kurane *et al.*¹⁴⁾ meso-DAP was determined by thinlayer chromatography (TLC). TLC was performed on a cellulose plate with the following system; methanol-distilled water-6N-HCl-pyridine = 80:26:4:10.¹⁵⁾

Isolation and determination of mycolic acid. Bacterium JTS-131 was cultured in L-broth for 3 days at 28°C with shaking. Isolation of mycolic acid from cell walls was carried out by the method of Yano *et al.*¹⁶⁾ After

trimethylsilylation, the resultant trimethylsilyl esters were analyzed by GC mass spectrometry (Hitachi M-80 GC mass spectrometer) with a glass column (1 m $\times$ 3 mm i.d.) packed with 1% Silicone OV-1 on Chromosorb W (AW, DMCS, 80~100 mesh).

Media. Medium-A, containing (g/liter) $(\text{NH}_4)_2\text{SO}_4$ 2.0, K_2HPO_4 2.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.2, and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01, and adjusted to pH 7.2 was used as the transformation basal medium. Medium-B, containing sodium succinate (5 g/liter) in medium-A, was used as the transformation medium. L-Broth, containing (g/liter) Bacto-trypton (Difco) 10.0, Bacto-yeast extract (Difco) 5.0, NaCl 5.0, and glucose 1.0, and adjusted to pH 7.0, was used for cell preparation for *meso*-DAP, mycolic acid and the plasmid. L-Agar, containing 1.5% of agar in L-broth, was used as the stock medium.

Preparation of L-agar plate containing cis-abienol. An L-agar plate, containing uniformly dispersed *cis*-abienol (CAL-plate) was prepared according to the method of Peterson *et al.*¹⁷⁾ Two grams of *cis*-abienol in 10 ml of 1% (w/v) Tween-60, autoclaved for 15 min at 121°C and then cooled to about 70°C, was added to separately sterilized Waring vessels containing 200 ml of cooled, molten L-agar. After blending for 2 min, the mixture (5 ml) was poured into a sterile plate in which 10 ml of L-agar had already hardened. It was necessary to remove foam from the surface of the plate with a gas burner for a few seconds.

A CAA-plate and CAB-plate were also prepared by the same procedure using medium-A and medium-B, respectively.

Curing experiment. Bacterium JTS-131 was inoculated into a test tube containing 5 ml of L-broth and incubated for 40 hr at 28°C. The cells were transferred to fresh L-broth containing mitomycin C (1.0 $\mu\text{g/ml}$) to a cell concentration of $10^5 \sim 10^6$ per ml and incubated at 28°C until full growth had occurred ($3 \sim 4 \times 10^9$ cells/ml). After dilution, the resulting culture was incubated on an L-plate. To select the cured strain, single colonies isolated from the L-plate were examined for the transformation ability using basal medium-A (5 ml/test tube), containing 0.1% *cis*-abienol and 0.01% Tween-60. After 48~60 hr incubation, the culture was acidified to pH 3.0 and extracted with ethyl acetate. The extract was analyzed by TLC and GC-MS.

Microbial transformation of cis-abienol, sclareol and their transformation products. Compound **A-1** [(12Z)-labda-12,14-dien-18-ol], compound **A-2** [(12Z)-labda-12,14-dien-18-oic acid] and compound **A-3** [(12Z)-labda-12,14-dien-18-oic acid methylester] were obtained as the transformation products from *cis*-abienol with bacterium JTS-131.¹⁾ Compound **A-4** [labda-12,14-dien-17 α -ol] was obtained as the transformation product from *cis*-abienol with *N. restricta* JTS-162.¹⁸⁾ Compound **S-1** [13 β -hydroxylabd-14-en-18-oic acid] and compound **S-2** [13 β -

hydroxylabd-14-en-18-oic acid methylester] were also obtained as the transformation products from sclareol with bacterium JTS-131.¹⁾ Compound **S-3** [labd-14-en-13 β ,18-diol] was prepared by the reduction of compound **S-1** using LiAlH_4 .¹⁾ Ten mg of each compound described above was added to 5 ml of medium-A or medium-B containing the cured strain (about $10^8/\text{ml}$) and 0.5 mg of Tween-60. After 60 hr incubation, the resulting culture was acidified to pH 3.0 and extracted with ethyl acetate. The extract was analyzed by TLC and GC-MS.

Thin layer chromatography (TLC). The sample was spotted on a Silica gel G plate (Merck) and developed with *n*-hexane-ethyl acetate (7:3 or 5:5, v/v). After evaporation of the organic solvents, the compounds were detected by spraying *p*-anisaldehyde (5%) and H_2SO_4 (5%) in 90% ethanol onto the plate and heating it at 120°C for 3 min.

Gas chromatography-mass spectrometry (GC-MS). GC-MS was performed under the following conditions, using a Hitachi M-80 GC mass spectrometer. The column was a glass tube (1 m $\times$ 3.2 mm i.d.) packed with 10% Silicone OV-101 on Chromosorb W (AW, DMSC, 60~80 mesh) or 2% Silicone XE-60 on Chromosorb W (AW, DMCS, 60~80 mesh).

Preparation of crude plasmid DNA. Plasmid DNA for electrophoresis was prepared by the method of Hansen and Olsen.¹⁹⁾ Cells grown in L-broth were suspended in 25% sucrose solution, and lysed by the addition of lysozyme, ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl acetate (SDS) followed by heat pulsing at 55°C. The clear viscous lysate thus obtained was subjected to alkaline denaturation and then salt-precipitation to remove chromosome-membrane complexes. Plasmid DNA was concentrated with polyethyleneglycol and dissolved in a small volume of buffer containing 50 mM Tris, 5 mM EDTA and 50 mM NaCl at pH 8.0 (TES buffer).

CsCl-ethidium bromide centrifugation. Further purification of crude plasmid DNA was carried out by centrifugation in CsCl-ethidium bromide solution according to the procedure of Yano *et al.*²⁰⁾

Agarose gel electrophoresis. Agarose gel electrophoresis was performed according to the method of Kado and Liu²¹⁾ in a horizontal apparatus (13.0 $\times$ 13.5 $\times$ 0.5 cm) of 0.5 and 0.7% agarose (Sigma type II) at 50 V. The gel was stained with ethidium bromide in water (0.5 $\mu\text{g/ml}$) for 30 min. The gel was visualized on a transilluminator model C-62 (Ultra-Violet Products Inc., San Gabriel, Calif.) and photographed on Polaroid 667 film, using UV and orange filters.

TABLE I. TAXONOMICAL PROPERTIES OF BACTERIUM STS-131

<p>Rods and cocci (0.5~0.7 by 5~15 μm after 12 hr incubation, 0.5~0.7 by 0.5~1.5 μm in old cultures). Fragmentation begins in the center of a colony after 12~14 hr incubation. Microcysts are common in old cultures. Gram-positive. Non-motile. Partially acid fast.</p> <p>Nutrient agar colonies (5 days); 0.5~1.5 mm, pasty and mucoid, pinkish gray with entire margins. Growth restricted on nutrient gelatin; no liquefaction.</p> <p>Acid from D-fructose, D-glucose, glycerol, sorbitol, maltose, mannitol, mannose, sucrose, trehalose and dextrin.</p> <p>Carbon utilization; D-alanine, L-alanine, D-fructose, D-glucose, glycerol, maltose, mannitol, mannose, malate, lactate, sebacic acid, adenine, inuline, adipate, gluconate, paraffin, sodium acetate, sodium citrate, sodium pyruvate, sodium succinate and trehalose.</p> <p>Urease present. Phosphatase present. Nitrite not produced from nitrate in nitrate broth. Nitrite produced from nitrate in succinate-nitrate broth.</p> <p>Litmus milk was alkaline, no coagulation.</p> <p>Adenine, esculin, and Tween-20, 40 and 60 hydrolysed. Casein not hydrolysed.</p> <p>Growth temperature; 5~40°C. Optimum; 28°C. Growth pH; 6~10. Optimum; 7.5. Not inhibited by 7% NaCl. Sensitive to penicillin (5 i.u. discs.). Lysozyme sensitive. GC content; 63.0 mol% (T_m).</p>

RESULTS

Taxonomical properties of cis-abienol transformable bacterium JTS-131

The morphological and physiological properties of soil bacterium JTS-131 are summarized in Table I. This bacterium was gram-positive, non-endospore forming, rod-filamentous, partially acid-fast, non-motile and aerobic, and had *meso*-diaminopimelic acid (*meso*-DAP) in the cell wall. According to Yamada and Komagata,²²⁾ and Komura *et al.*,²³⁾ *meso*-DAP as the principal amino acid in the cell wall was found in members of *Corynebacterium*, *Brevibacterium* and *Nocardia*. Furthermore, this strain contained free mycolic acids with 36 to 48 carbon units. It is known that the mycolic acids of *Nocardias* contain some 50 carbon atoms.^{24,25)} From these results, we tentatively classified this microorganism into the genus *Nocardia*.

From the results of fragmentation after 12~14 hr incubation, this strain seemed to fall into "morphological group I of *Nocardia*," with reference to the 8th edition of Bergey's Manual of Determinative Bacteriology. This bacterium did not hydrolyse casein and produced nitrite in succinate-nitrate broth, but not in nitrate broth. This suggests that the bacterium potentially has nitrate reductase activities as has been reported by Komura *et al.*²⁶⁾ From other properties summarized in

Table I, bacterium JTS-131 was identified as *Nocardia erythropolis* (Gray and Thornton) according to the 8th edition of Bergey's Manual. However, in 1980, *Nocardia erythropolis* was classified as *Rhodococcus erythropolis* in the "Approved list of bacterial names" presented by the Ad Hoc Committee of the Judicial Commission of the International Committee on Systematic Bacteriology (ICBS).²⁷⁾ Bacterium JTS-131 showed the same physiological and biochemical characteristics as ATCC 4277, the type strain of *Rhodococcus erythropolis*. From these results, we identified bacterium JTS-131 as *Rhodococcus erythropolis*.

Identification of the cured strain

To examine whether the transformation of *cis*-abienol is determined by the plasmid or not, curing experiments were carried out (see METHODS). On a CAL-plate and CAB-plate, bacterium JTS-131 could grow as well as on an L-plate, but no clear zones were not formed around the colonies even if the incubation was carried out for 3~4 weeks. On a CAA-plate, bacterium JTS-131 could not grow even if the cells were plated from an L-plate by the replica method. Based on these results, the cured strain was obtained by incubation of each colony, using medium-A (5 ml/test tube) containing *cis*-abienol and Tween-60. After 60 hr incubation, the resulting culture was acidified

to pH 3.0 and extracted with ethyl acetate. The extract was analyzed by TLC and GC-MS.

As a result, three colonies (JTS-13101, -13102 and -13103; frequency: 3/430) were obtained on mitomycin C treatment. The three strains thus obtained could not grow on medium-A containing *cis*-abienol. The concentration of added *cis*-abienol in the resulting culture was not changed even if prolonged incubation (~120 hr) was carried out. *cis*-Abienol was utilized by the bacterium JTS-131 and not detected in the culture broth after 120 hr incubation under the same conditions.

Microbial transformation of cis-abienol, and compounds A-1, A-2 and A-3 by the cured strains

Compounds A-1, A-2 and A-3 were not utilized by these three cured strains, and the concentration of each compound added to medium-A and medium-B was not changed. The three cured strains slowly utilized *cis*-abienol in medium-B without accumulating transformation products. After 120 hr incubation, the three cured strains had utilized only 12% of added *cis*-abienol, though the wild strain utilized all *cis*-abienol added (Fig. 1). In the presence of EDTA, added to a 12-hr-old culture, the three cured strains could not accumulate any transformation product from *cis*-abienol, but the wild strain accumulated compounds A-1, A-2 and A-3 from *cis*-abienol in

the culture broth. Compound A-4 was utilized by both the wild strain and the cured strains in medium-A and medium-B.

Microbial Transformation of Sclareol, and compounds S-1, S-2 and S-3 by the cured strains

These three cured strains could not utilize sclareol or compounds S-1, S-2 and S-3 in medium-A and medium-B. Under the same conditions, wild strain JTS-131 could utilize all these four compounds.

Gel electrophoresis of plasmids

Figure 2 shows typical agarose gel electrophoresis patterns of plasmids prepared from JTS-131 and JTS-13101. Wild strain JTS-131 showed plasmid pCA4. But cured strain JTS-13101 had no plasmid. The other two cured strains also indicated the absence of a plasmid.

Analyses of plasmids in Rhodococcus erythropolis JTS-131 and Nocardia restricta JTS-162

To compare the labdane transformation abilities and the plasmids of the two *cis*-abienol transformable bacteria, *Rhodococcus erythropolis* JTS-131 and *Nocardia restricta* JTS-162, the plasmids from these two strains were compared in 0.5% agarose. As shown in Fig. 3, pCA4 was larger than pCA1, pCA2 and pCA3 obtained from *N. restricta* JTS-162.

FIG. 1. Time Course of Utilization of *cis*-Abienol by *R. erythropolis* JTS-131 and its Cured Strain, JTS-13101.

Ten mg of *cis*-abienol was added to medium-B (5 ml/test tube) containing 0.5 mg Tween-60 and cells. Incubation was carried out at 28°C with mechanical shaking.

Symbols: ○—○, JTS-131; ●—●, JTS-13101.

FIG. 2. Agarose Gel Electrophoresis of Plasmids from *R. erythropolis* JTS-131 and its Cured Strain, JTS-13101. A, plasmid of JTS-131; B, plasmid of JTS-13101.

FIG. 3. Agarose Gel Electrophoresis of Plasmids from *N. restricta* JTS-162 and *R. erythropolis* JTS-131.

A, *N. restricta* JTS-162; B, *N. restricta* JTS-162 plus *R. erythropolis* JTS-131; C, *R. erythropolis* JTS-131.

The DNA bands of these plasmids shown in Figs. 2 and 3 are covalently closed circular (ccc) DNA because of the same mobility on agarose gels as more purified cccDNA on CsCl-ethidium bromide equilibrium density gradients.¹⁹⁾

DISCUSSION

In the previous paper,¹⁾ we reported the microbial transformation of *cis*-abienol and sclareol by soil bacterium JTS-131. These two labdanes were oxidized by this bacterium and converted to hydroxymethyl, carboxyl acid and methylester derivatives successively.

In the other papers,^{2,3)} we reported that the soil bacterium, *Nocardia restricta* JTS-162, transformed *cis*-abienol and sclareol. By the bacterium, the C-8 and C-17 positions of *cis*-abienol were firstly dehydrated, and then the C-18 methyl was oxidized stepwisely and the A-ring was split successively. It was considered that this C-18 methyl oxidation and A-ring splitting were determined by the plasmid (pCA1) in bacterium JTS-162.

In this study, we identified bacterium JTS-131 as *Rhodococcus erythropolis* from the morphological characteristics. This bacterium is widely distributed in soil in Japan as a major

FIG. 4. Plasmid-determined Transformation Pathways for *cis*-Abienol and Sclareol in *R. erythropolis* JTS-131.

gram-positive bacterium.²⁸⁾ It was also reported that *R. erythropolis* (*N. erythropolis*) could metabolize organic compounds such as sterols,^{29,30,31)} aromatic compounds³²⁾ and phthalates.¹⁴⁾ However, it has not been reported that such abilities to metabolize these organic compounds are determined by the plasmid in *R. erythropolis* (*N. erythropolis*). In this study, from the results of curing experiments (Fig. 2), it was elucidated that the C-18 methyl oxidation of *cis*-abienol and sclareol by *R. erythropolis* JTS-131 was determined by the plasmid, pCA4 (Fig. 4). In the *cis*-abienol transformation by the cured strains from JTS-131, 12% of *cis*-abienol added was utilized in medium-B after 120 hr incubation (Fig. 1). However, these cured strains could not utilize *cis*-abienol in medium-A. And they also could not utilize compounds A-1, A-2 and A-3 in both medium-A and medium-B. In the previous work,¹⁾ it was demonstrated that conversion products from *cis*-abienol were ob-

tained only by the addition of metabolic inhibitors, such as EDTA. In this study, these cured strains could not accumulate further conversion products from *cis*-abienol in the presence of EDTA. These results may indicate that *cis*-abienol is also utilized by other metabolic pathways different from the transformation pathway previously described.¹⁾ The utilization of compound **A-4** by this strain may indicate the presence of a transformation pathway containing compound **A-4** as the metabolic intermediate.

The two plasmids, pCA1 and pCA4, which were considered to deremine the C-18 oxidation and A-ring splitting in *N. restricta* JTS-162 and C-18 oxidation in *R. erythropolis* JTS-131, respectively, were analyzed by electrophoresis on 0.5% agarose gel. As shown in Fig. 3, the two plasmids showed different mobilities, and pCA4 was larger than pCA1. It is interesting to study the relationship between these two plasmids. Studies on functions and the molecular nature of these two plasmids, pCA1 and pCA4, are now in progress.

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REFERENCES

- 1) T. Hieda, Y. Mikami, Y. Obi and T. Kasaki, *Agric. Biol. Chem.*, **47**, 243 (1983).
- 2) T. Hieda, Y. Mikami, Y. Obi and T. Kasaki, *Agric. Biol. Chem.*, **46**, 2249 (1982).
- 3) T. Hieda, Y. Mikami, Y. Obi and T. Kasaki, *Agric. Biol. Chem.*, **46**, 2477 (1982).
- 4) T. Hieda, Y. Mikami, Y. Obi and T. Kasaki, *Agric. Biol. Chem.*, **46**, 3055 (1982).
- 5) K. Yamada and K. Komagata, *J. Gen. Appl. Microbiol.*, **18**, 399 (1972).
- 6) Society of American Bacteriologists, Committee of Bacteriological Technique (Chairman; M. J. Pelcer, Jr.) "Manual of Microbiological Methods," McGraw-Hill, New York, 1956.
- 7) T. Toda, *Nihon Iji Shimpō*, **283**, 113 (1928).
- 8) F. Neelsen, *Centr. Med. Wisse.*, **21**, 497 (1883); cited from "Biseibutsu no Bunrui to Dotei (Classification and Identification of Microorganisms)," University of Tokyo Press Co., Tokyo, 1975, p. 218.
- 9) R. Y. Stanier, N. J. Palleroni and M. Doudoroff, *J. Gen. Microbiol.*, **43**, 159 (1966).
- 10) T. Hasegawa, "Biseibutsu no Bunrui to Dotei (Classification and Identification of Microorganisms)," University of Tokyo Press, Tokyo, 1975, p. 222.
- 11) R. E. Gordon and J. M. Mihm, *J. Gen. Microbiol.*, **21**, 736 (1959).
- 12) K. Yamada and K. Komagata, *J. Gen. Microbiol.*, **16**, 103 (1970).
- 13) J. Marmur, *J. Mol. Biol.*, **3**, 208 (1961).
- 14) R. Kurane, T. Suzuki, Y. Takahara and K. Komagata, *Agric. Biol. Chem.*, **41**, 1031 (1977).
- 15) J. L. Stanneck and G. D. Roberts, *Appl. Microbiol.*, **28**, 226 (1974).
- 16) I. Yano and K. Saito, *FEBS Lett.*, **21**, 215 (1972).
- 17) G. E. Peterson, H. L. Lewis and J. R. Davis, *J. L. Research*, **3**, 275 (1962).
- 18) T. Hieda, Y. Mikami and Y. Obi, *Agric. Biol. Chem.*, **47**, 47 (1983).
- 19) J. B. Hansen and R. H. Olsen, *J. Bacteriol.*, **135**, 227 (1978).
- 20) K. Yano and T. Nishi, *J. Bacteriol.*, **143**, 552 (1980).
- 21) C. I. Kado and S. T. Liu, *J. Bacteriol.*, **145**, 1365 (1981).
- 22) K. Yamada and K. Komagata, *J. Gen. Appl. Microbiol.*, **16**, 103 (1970).
- 23) I. Komura, K. Yamada, S. Otsuka and K. Komagata, *J. Gen. Appl. Microbiol.*, **21**, 251 (1975).
- 24) L. Alshamaony, M. Goodfellow and D. E. Minnikin, *J. Gen. Microbiol.*, **92**, 188 (1976).
- 25) D. E. Minnikin and M. Goodfellow, "The Biology of the Nocardiae," ed. by M. Goodfellow, G. H. Brownell and J. A. Serrano, Academic Press, Inc., New York, N. Y., 1976, p. 160.
- 26) I. Komura, K. Yamada, S. Otsuka and K. Komagata, *J. Gen. Appl. Microbiol.*, **19**, 161 (1973).
- 27) The Ad Hoc Committee of the Judicial Commission of the ICBS, *Int. J. Syst. Bacteriol.*, **30**, 356 (1980).
- 28) K. Kato, Abstracts of Papers, Meeting of the Society of the Science of Soil and Manure, Japan, Kobe, April, 1976, p. 42.
- 29) D. Kluepfel and C. Vezina, *Appl. Microbiol.*, **20**, 551 (1970).
- 30) S. Sehgal and C. Vezina, *Appl. Microbiol.*, **20**, 875 (1970).
- 31) K. Singh, D. J. Marshall and C. Vezina, *Appl. Microbiol.*, **20**, 23 (1970).
- 32) R. B. Cain and N. J. Cartwright, *Biochim. Biophys. Acta*, **37**, 197 (1960).