Purification and Characterization of Crotonase from Clostridium acetobutylicum

(Received for publication, March 29, 1972)

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SUMMARY

An enzyme catalyzing the reversible hydration of crotonyl-CoA has been obtained in homogeneous form from Clostridium acetobutylicum. The bacterial hydrase has a molecular weight of 158,000 ± 3,000 as determined by sedimentation equilibrium. In contrast, the enzyme has a molecular weight of approximately 43,000 in 6 M guanidine hydrochloride in either the presence or absence of reducing agent. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate yields a molecular weight of 40,000, also about four-fold that of the native enzyme. Unlike bovine liver crotonase, the bacterial enzyme is specific for short chain fatty acyl-CoA substrates and is sensitive to high concentrations of crotonyl-CoA. It requires a complete coenzyme A thioester substrate for efficient catalysis. It is concluded that crotonase from C. acetobutylicum is composed of 4 subunit polypeptide chains, with each chain containing about 370 residues devoid of any attached carbohydrate, and that the 4 subunits of the native enzyme are combined through noncovalent interactions. There are, however, a number of similarities between the bacterial and mammalian enzymes, which suggest that the two may be structurally related.

Recent reports have yielded insight into the structure function properties of the bovine enzyme (2-4). The apparent uniqueness of the mammalian crotonase will respect to the other enzymes involved in fatty acid oxidation has led us to an investigation of the hydratase activity of a more primitive organism. We report here the purification and some of the properties of an enoyl-CoA hydratase from the obligate anaerobe Clostridium acetobutylicum, including its molecular weight, subunit structure, amino acid composition, and substrate specificity.

EXPERIMENTAL PROCEDURE

Materials—Sephadex G-200 and DEAE-Sephadex (Pharmacia) were swollen and equilibrated by the standard procedures recommended by the manufacturer. Purified guanidine hydrochloride was obtained from Heico and used without additional recrystallization. All other compounds were reagent grade and were used without further purification.

Crotonyl-CoA was prepared using crotonic anhydride (Eastman) and reduced coenzyme A (P and L Laboratories) by the method of Simon and Shemin (5). Crotonylpantetheine was prepared similarly using reduced pantetheine (Sigma). Purified α,β-unsaturated free fatty acids containing 6, 8, 10, 12, 14, and 16 carbon atoms were a gift from Dr. Salih Wakil. The CoA thioesters of each were prepared as described in an accompanying report (3) and were obtained as single chromatographic components prior to use.

Electrophoretic Analyses—Disc electrophoresis was performed with 6% polyacrylamide gels at pH 8.5 as previously described (6). The method of Panyim and Chalkley (7) was followed for electrophoresis (7%) separating gels) in 6.25 M urea, pH 3.2. The method of Shapiro et al. (8) as modified by Weber and Osborn (9) was used for the electrophoretic analysis of proteins in sodium dodecyl sulfate.

Ultracentrifugal Studies The meniscus depletion sedimentation equilibrium method of Yphantis (10) was used to determine molecular weights of crotonase in dilute salt solutions as well as in 6 M guanidine hydrochloride. The experimental procedures are essentially identical with those reported earlier from this laboratory (2). The apparent partial specific volume of crotonase was calculated from the amino acid composition (11).

Extinction Coefficients—The extinction coefficient for dilute salt solutions of C. acetobutylicum crotonase was determined by the ultracentrifugal method of Rubul and Stadlwagner (12). A solution of crotonase was dialyzed against 0.05 M Tris-Cl, pH 8, containing 0.1 M KCl and the absorbance of the solution deter-
minded with the dialysate as a blank. The absorbance was corrected for light scattering as previously described (13). These analyses gave $e_{280}^{1%}$ = 0.900.

Amino Acid Compositions—Amino acid analyses were performed with a Beckman model 120B automatic amino acid analyzer (14) equipped with Bio-Cal automatic sample injectors as described earlier (15). Triplicate hydrolysates were analyzed for each time interval.

The total half-cystine content was determined as cysteic acid analysis of acid hydrolysates of performic acid-oxidized crotonase (16). Tryptophan was estimated by the spectrophotometric method of Edelhoch (17).

Kinetic Assays—All assays were performed with a Cary model 15 spectrophotometer at 25° in 1.0-ml quartz cuvettes with a 1.0-cm path length. Initial rates of hydration were determined from the decrease in absorbance in the region of 250-290 nm due to hydration of the enoyl-CoA conjugated double bond as described earlier (3, 18).

**Purification of Enoyl-CoA Hydratase**

Growth of Bacteria and Preparation of Acetone Powder—C. acetobutylicum was grown anaerobically as previously described (19). Spores were provided by Dr. Irwin Fridovich. Cells were harvested (225 g) after reaching a growth plateau. One-half of the cells were mixed with 800 ml of acetone chilled with Dry Ice, and the mixture was blended in a Waring Blender for 2 min at high speed. The remaining cells were treated identically. After blending, the cell suspension was poured from the blender and 700 ml of acetone were mixed with the residue in the blender. All fractions were combined and stirred for 10 min in a beaker and then filtered through a large Buchner funnel. The residue was washed first with acetone (2 liters, $-5°$) and then ether (2 liters, $-5°$). Residual ether was removed under vacuum. A total of 56.5 g of powder were obtained from the 225 g of wet cells.

Preparation of Extracts—Ten grams of acetone powder were suspended in 500 ml of 0.02 M potassium phosphate, 0.003 M potassium EDTA, pH 7.5, and stirred for 4 h for 24 hours. Insoluble material was removed by centrifugation (Sorvall RC-2; type GSA rotor) for 1 hour at 9000 rpm.

Acid Heat Treatment—The clear supernatant solution was adjusted to pH 5 with 1 M acetic acid. The solution was then heated to 60° with stirring for 4 min, cooled, and adjusted to pH 7 with 1 M potassium bicarbonate. Denatured protein was removed by centrifugation for 45 minutes at 9000 rpm.

Acetone Precipitation—The supernatant solution from the previous step was cooled to 0° in an ice bath. An equal volume of acetone ($-5°$) was gradually added while maintaining the temperature between 0° and $-5°$. As is observed with the bovine liver enzyme, crotonase from C. acetobutylicum is rapidly and irreversibly denatured at temperatures much above 0° in the presence of acetone. Care must be taken to insure that acetone is not added too rapidly. The suspension was added to previously cooled centrifuge bottles and spun at 9000 rpm for 40 min at $-5°$. The pellet was dissolved in 50 ml of the phosphate-EDTA buffer used for extraction of the acetone powder, concentrated by vacuum dialysis to a volume of 10 ml, and dialyzed against 4 liters of the same buffer at $4°$ for 18 hours. Insoluble material formed during dialysis was removed by centrifugation.

Sephadex G-200 Chromatography—The dialyzed solution (10 ml) from the preceding step was chromatographed on Sephadex G-200 at 4°. The column ($2.5 \times 85$ cm), equilibrated with 20 mM phosphate, 3 mM EDTA, pH 7.4, was developed at a flow rate of 24 ml per hour and 6-ml fractions were collected. The elution profile obtained is shown in Fig. 1. Fractions were assayed for crotonyl-CoA hydratase activity as indicated by the open circles. Fractions 32 to 42 were pooled and contained 90% of the activity applied to the column.

Chromatography on DEAE-Sephadex—The pooled fractions from the preceding step were dialyzed exhaustively against 0.05 M Tris-HCl, pH 8.0, at 4°. This solution (66 ml) was applied to a column (0.9 $\times$ 15 cm) of DEAE-Sephadex which had been equilibrated at 4° with 0.05 M Tris-HCl, pH 8.0. After the column was washed with several column volumes of starting buffer, elution was effected with a linear gradient composed of 250 ml of 0.05 M Tris-HCl, pH 8.0, and 250 ml of 0.40 M Tris-HCl, pH 8.0. The elution profile for total protein and the activity peak observed are shown in Fig. 2. The well separated activity peak was found to contain a single component as described below.
**TABLE I**

| Step                                      | Volume | Protein concentration | Total protein | Units$^b$ per mg | Total activity | Yield | Fold purification |
|-------------------------------------------|--------|-----------------------|---------------|-----------------|---------------|-------|------------------|
| 1. Extraction from 10 g of acetone powder$^c$ | 401    | 2.20                  | 882           | 47              | 41,464        | 100   | 1                |
| 2. Acid-heat                               | 421    | 1.75                  | 737           | 61.6            | 45,377        | 100   | 1.31             |
| 3. 0 to 50% acetone precipitate            | 50     | 3.30                  | 165           | 195.9           | 22,324        | 78    | 4.17             |
| 4. G-200 Chromatography$^d$                | 66     | 0.19                  | 12.5          | 2,310.0         | 28,877        | 69.6  | 49.15            |
| 5. DEAE pool and concentrate               | 8      | 0.56                  | 4.48          | 6,155           | 27,575        | 68.5  | 131.0            |

$^a$ Protein determined by biuret.
$^b$ 1 unit = 1 μmole hydrated per min per mg of protein.
$^c$ Obtained from approximately 40 g of wet cells.
$^d$ Using $e_{280} = 0.89.$

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**Fig. 3.** Polyacrylamide gel electrophoresis of *Clostridium acetobutylicum* crotonase. A, tracing of the gels obtained following electrophoresis of the protein by the discontinuous system (6) at pH 8.5; B, the pattern obtained when crotonase was subjected to electrophoresis in the presence of urea as described in the text. *Insets,* photographs of the respective gels.

**Fig. 4.** Sedimentation equilibrium of *Clostridium acetobutylicum* crotonase. A, 0.001% crotonase in 0.1 m KCl, 0.02 m potassium phosphate, pH 7.5, at 17,000 rpm; B, 0.001% crotonase in 6 m guanidine hydrochloride, pH 4.5, at 40,000 rpm; C, 0.001% crotonase in 6 m guanidine hydrochloride, 0.14 m β-mercaptoethanol, pH 4.5, after reduction at pH 8.0 with 0.14 m mercaptoethanol, at 36,000 rpm. *Ordinates,* natural logarithms of the fringe displacement; *abscissas,* squares of the radial distance; *arrows,* position of the bottom of the cell.

**EXPERIMENTAL RESULTS**

**Purification of Crotonase**—The enzyme was isolated from an acetone powder of *C. acetobutylicum* cells by a sequence of steps involving (a) solubilization, (b) acidification and heat denaturation of other proteins, (c) 0 to 50% acetone fractionation, (d) gel filtration on Sephadex G-200, and (e) DEAE-ion exchange chromatography. Crotonase (4.48 mg) was obtained from 10 g of acetone powder by this technique (Table I). This represents a 131-fold purification and a 66.5% yield based upon the crude extract.

**Purity and Stability**—Crotonase prepared by the method described lost activity when stored in buffer solutions at 4°C, or when frozen. Lyophilization resulted in negligible loss of enzymatic activity, therefore the protein was customarily stored in this manner. The crotonase prepared by this procedure appeared to be pure as judged by standard electrophoretic methods. The protein migrated as a single component on disc gel electrophoresis at pH 8.5 and on polyacrylamide gel electrophoresis at pH 3.2 in 6 m urea (Fig. 3). In addition, as discussed below, a single component was obtained on electrophoresis in sodium dodecyl sulfate. Clearly, the bacterial enzyme behaves as a homogeneous protein in both the native and denatured states.

**Molecular Weights**—The weight-average molecular weight of the hydrazide in dilute salt solutions was obtained from sedimentation equilibrium studies. The results are shown in Fig. 4. The linear relationship between the natural logarithm of the fringe displacement and the square of the distance from the center of rotation is representative of a homogeneous, monodisperse system. The molecular weight obtained from the slopes of these lines was found to be 158,000 ± 3,000, based upon a partial specific volume of 0.742 as calculated from the amino acid composition (11).

Similar studies were performed in 6 m guanidine hydrochloride both in the absence (Fig. 4B) and presence (Fig. 4C) of mercaptoethanol. A molecular weight of 43,700 ± 200 was obtained with both systems. Clearly, the presence of reducing agent had no effect upon sedimentation equilibrium. This observation suggests that there are no inter-chain disulfide bonds present.

Molecular weight determinations of proteins in concentrated guanidine hydrochloride are complicated by the possibility that preferential interactions between the salt and the protein may occur resulting in the exclusion of water from the normal hy-
drated species (21). An average partial specific volume decrease of 0.01 ml per g has been noted for myosin (22), whereas several other proteins display no such preferential interaction effect (23, 24). Assuming no change in the partial specific volume of crotonase, the data in Fig. 4 B and C yield a molecular weight of 43,700 both with and without reducing agent present. Values of 40,700, on the other hand, are obtained if the partial specific volume for the protein is assumed to decrease by 0.01 ml per g in guanidine hydrochloride.

The subunit molecular weight was also examined by gel electrophoresis in sodium dodecyl sulfate. The results obtained are shown in Fig. 5. The relationship between the logarithm of molecular weight and the relative mobility of the proteins is linear for this molecular weight range, as is expected with 5% polyacrylamide. The molecular weight obtained for C. acetobutylicum crotonase is 40,000 ± 500. This is in good agreement with the values obtained from ultracentrifugal studies, and tends to suggest that the preferential interaction assumptions made for guanidine hydrochloride effects are necessary and valid.

Since the molecular weight in either 6 M guanidine hydrochloride or 0.1% sodium dodecyl sulfate is quite close to one-fourth of that in dilute buffer solution, it is concluded that C. acetobutylicum crotonase contains 4 subunit polypeptide chains.

**Amino Acid Composition**—The amino acid composition for the hydrazine is presented in Table II. The composition determined earlier for the mammalian enzyme (2) is included for comparative purposes. The values obtained for isoleucine and valine are assumed to be represented by the 96-hour hydrolyses, while those for serine and threonine were estimated by extrapolation to zero-time for hydrolysis. Half-cystine and tryptophan were determined independently as described under “Experimental Procedure.” The calculated values for all other amino acids are the average of the values from the 24-, 48-, and 96-hour hydrolyses. The phenol-sulfuric acid test for carbohydrate content was kindly performed by Dr. Ian Tmyer, and indicated that the protein was devoid of neutral sugars. In addition, the recovery of amino acids accounts for the weight of protein hydrolyzed, suggesting that the enzyme does not contain associated lipid or carbohydrate.

**Catalytic Properties**—The substrate specificity of the bacterial crotonase was determined with the series of CoA derivatives of even numbered trans-α,β-unsaturated fatty acids from 4 to 16 carbon atoms in length. In contrast to the broad specificity of bovine liver crotonase (3, 30), the hydrase obtained from C. acetobutylicum was found to be of limited specificity. Only crotonyl-CoA and hexenoyl-CoA were hydrated and higher analogs were not substrates. Activity towards C2 to C6 enoyl-CoA substrates was found, however, in the crude extracts, which indicates that more than one hydrase is present in the organism. The rate of hydration of crotonyl-CoA as a function of its concentration is presented in Fig. 6. Values for $V_{max}$ and $K_m$ of 6.5 × 10⁶ moles per min per mole and 3 × 10⁻⁵ M, respectively, were obtained from double reciprocal plots (Fig. 7). The rate of hydration of hexenoyl-CoA, unlike that of crotonyl-CoA, obeys normal Michaelis-Menten kinetics and analysis of a double reciprocal plot of the data is linear with no evidence of substrate inhibition. Values of 3.9 × 10⁶ min⁻¹ and 1.3 × 10⁻⁴ M were obtained for $V_{max}$ and $K_m$, respectively.

![Graph](https://example.com/graph.png)  
**Fig. 5.** Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Samples of reduced, dissociated proteins were subjected to electrophoresis on 5% gels as described by Weber and Osborn (9). The molecular weight for each protein is shown on the plot of log molecular weight versus mobility. The values obtained for isoleucine and valine are assumed to be represented by the 96-hour hydrolyses, while those for serine and threonine were estimated by extrapolation to zero-time for hydrolysis. Half-cystine and tryptophan were determined independently as described under "Experimental Procedure." The calculated values for all other amino acids are the average of the values from the 24-, 48-, and 96-hour hydrolyses. The phenol-sulfuric acid test for carbohydrate content was kindly performed by Dr. Ian Tmyer, and indicated that the protein was devoid of neutral sugars. In addition, the recovery of amino acids accounts for the weight of protein hydrolyzed, suggesting that the enzyme does not contain associated lipid or carbohydrate.

| Amino Acid     | C. acetobutylicum | Bovine Liver⁷ |
|----------------|------------------|--------------|
|                | mol wt 165,000   | mol wt 21,000 |
| Lysine         | 134             | 127          |
| Histidine      | 13              | 6            |
| Arginine       | 59              | 49           |
| Aspartic acid  | 150             | 114          |
| Threonine      | 80              | 77           |
| Serine         | 73              | 72           |
| Glutamic acid  | 157             | 99           |
| Proline        | 45              | 55           |
| Glycine        | 137             | 146          |
| Alanine        | 150             | 209          |
| Half-cystine   | 30              | 32           |
| Valine         | 113             | 109          |
| Methionine     | 32              | 45           |
| Isoleucine     | 113             | 99           |
| Leucine        | 114             | 124          |
| Tyrosine       | 24              | 30           |
| Phenyldalanine | 37              | 70           |
| Tryptophan     | 16              | 9            |

Tom total no. of residues 1481 309 1561 262

⁷ Taken from Hass and Hill (2).

These values were extrapolated to zero time hydrolysis.

Measured as cysteic acid after performic acid oxidation.

These values assumed to be those given by 96-hour hydrolysis.

Detemined separately as described in the text.
Crotonyl COA Concentration, moles per liter x 10^5

**FIG. 6.** Hydration of crotonyl-CoA by Clostridium acetobutylicum. Crotonase (2 ng) was added to reaction mixtures containing 3.3 x 10^{-2} M Tris HCl buffer, pH 7.5, 5 x 10^{-1} M egg albumen, 5 x 10^{-4} M EDTA, and crotonyl-CoA at the concentrations indicated. Initial rates of hydration were measured spectrophotometrically at 280 nm.

**FIG. 7.** Double reciprocal plot for crotonyl-CoA hydration. The data correspond to those shown in Fig. 6.

The remarkable sensitivity to high concentrations of crotonyl-CoA was entirely unexpected since no such substrate inhibition was found with bovine liver crotonase (3, 30). No inhibition was observed with hexenoyl-CoA, even at levels at which the enzyme is totally inactivated by crotonyl-CoA.

A strict requirement for a complete coenzyme A structure is displayed by the bovine hydrase (4, 30). The catalytic behavior of the bacterial enzyme toward crotonyl-pantetheine was therefore examined. As with bovine liver crotonase, crotonyl-pantetheine is hydrated by the bacterial enzyme at a very low rate and the rate at low substrate concentrations was enhanced about 10-fold by either CoA, acetyl-CoA, or ATP (4).

**TABLE III**

Relative amino acid compositions of bacterial and bovine crotonases

| Amino acid       | Bacterial | Bovine |
|------------------|-----------|--------|
| Lysine           | 12        | 11     |
| Histidine        | 1         | 1      |
| Arginine         | 5         | 4      |
| Aspartic acid    | 13        | 10     |
| Threonine        | 7         | 7      |
| Serine           | 6         | 6      |
| Glutamic acid    | 14        | 17     |
| Proline          | 4         | 4      |
| Glycine          | 12        | 12     |
| Alanine          | 10        | 9      |
| Methionine       | 3         | 4      |
| Isoleucine       | 10        | 8      |
| Leucine          | 10        | 10     |
| Tyrosine         | 2         | 2      |
| Phenylalanine    | 3         | 6      |
| Tryptophan       | 1         | 1      |

Number of Residues: 129 (Bacterial), 133 (Bovine)

The amino acid compositions shown are based upon a molecular weight of 14,000 for a potential "ancestral" chain length as described in the text.

**DISCUSSION**

Properties of *C. acetobutylicum* Crotonase—The present studies describe the preparation and partial characterization of a crotonase from *C. acetobutylicum*. The protein is judged to be homogeneous based upon analysis by sedimentation equilibrium in dilute salt or 6 M guanidine hydrochloride solutions and by polyacrylamide gel electrophoresis under a variety of conditions.

The native enzyme has a molecular weight of 185,000 ± 3,000 as determined by sedimentation equilibrium. A molecular weight of approximately 40,000 is observed under conditions which are known to cause separation of polypeptide chains. Clearly, these data indicate that the crotonase of *C. acetobutylicum* is a tetramer, being comprised of subunits of molecular weight of about 40,000 which are combined by noncovalent bonds.

The hexameric bovine liver enzyme has been shown (2) to have a compact globular structure (f/fmin = 1.2). The fact that the bacterial enzyme with a molecular weight of 185,000 eluted from the G-200 column (Fig. 1) with a partition coefficient almost identical with that of the mammalian enzyme provides indirect evidence that *C. acetobutylicum* crotonase is also a typically globular protein. A direct proof of this hydrodynamic property awaits further study.

The substrate specificity of the bacterial hydrase is considerably different from that of the bovine liver enzyme. The bovine enzyme acts upon C4 to C6 substrates and appears to be the only hydrase in liver. In contrast the bacterial enzyme displays a strict preference for short chain substrates and appears to be but one of several hydratases. It is interesting to consider that the extremely high turnover rate and sensitivity to substrate inhibition displayed for crotonyl-CoA may be a reflection of the unique fermentation mode of acetobutylicum and related forms of Clostridia, and may affect the distribution of end products (31).

Comparison of Crotonase from *C. acetobutylicum* and Bovine Liver—Comparison of the molecular and catalytic properties of the bovine and bacterial crotonases allows a preliminary analysis of the evolution of the structure and function of the enoyl-CoA hydrases. The evolutionary lines leading to mammals and to bacteria diverged approximately one billion years ago. Had the bacterial enzyme and bovine enzyme been derived from a common ancestral crotonase, fairly extensive similarities in the kinetic and molecular parameters of these enzymes might be...
expected. Even though the subunit molecular weights are significantly different (~28,000, bovine; ~40,000, bacterial), there are a number of similarities in other properties of these enzymes which suggest that they may be of common genetic origin.

The most striking similarity between the two enzymes is their amino acid composition (Table II). The molecular weights of the 2 subunits are very close to integral multiples of 14,000. When the composition of each chain is based on this potential "ancestral" chain length (Table III) the similarities become more apparent. Particularly noticeable are the relatively high levels of half-cystine found in each and the low levels of histidine. Metzger et al. (32) have devised a means of comparing proteins based upon amino acid compositions. The difference index (D.I.) of two proteins is defined as 50 \times \Delta, where \Delta is the sum of the absolute values of the differences in mole fractions of each amino acid observed in the proteins. Comparison of the bovine and clostridial enzymes by this means yields a D.I. of 8.41. This value indicates that these two hydratases are more closely related than the \alpha and \beta chains of the hemoglobin (D.I. = 13.9) and bovine tryptophan and chymotrypsigen (D.I. = 13.8) and compare favorably with the values (7.4 to 11.5) found when relating heavy and light immunoglobulin chains. By this means they are less similar than the human triosephosphate dehydrogenase compared with similar enzymes from rabbit, chicken, sturgeon, and Escherichia coli (D.I. = 2.9, 3.3, 5.4, and 7.5, respectively). The D.I. for bovine and bacterial crotonases is significantly lower than that generally observed between functionally unrelated proteins (D.I. = 15–40) suggesting that these enzymes evolved from a common ancestral protein.

In addition to the marked similarity in amino acid composition between the bovine and bacterial enzymes, several of their kinetic properties are homologous. Both enzymes are extremely efficient and exhibit maximal turnover rate with crotonyl-CoA as substrate. The \(K_m\) values of each are extremely similar as well. In addition, an apparent equilibrium constant for the reaction between the bovine enzyme (18), as would be expected. Unlike other mammalian enzymes involved in \(\beta\) oxidation of fatty acids, both the bovine and bacterial crotonases exhibit a significant preference, \(i.e.,\) several orders of magnitude, for the CoA derivatives as compared with pantetheine derivatives. Interestingly, the addition of CoA, acetyl-CoA, or ATP to crotonylpantetheine greatly enhances the rate of hydration by both the bacterial and the bovine enzymes. This effect, attributed in the bovine system to substrate complementation (4, 30) rather than thiol transacylation as proposed earlier (18), may be of some significance which is as yet not fully understood, since it has been retained over approximately 10^8 years of independent evolution of these two enzymes.

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Purification and Characterization of Crotonase from Clostridium acetobutylicum
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J. Biol. Chem. 1972, 247:5266-5271.

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