Biosynthesis of Branched-Chain Amino Acids in Yeast: Correlation of Biochemical Blocks and Genetic Lesions in Leucine Auxotrophs

T. SATYANARAYANA, H. E. UMBARGER, AND GERTRUDE LINDEGREN¹

Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907

Received for publication 16 September 1968

The three enzymatic steps in the conversion of α -ketoisovalerate to α -ketoisocaproate were examined in wild-type and in leucine auxotrophic stocks of yeast. Procedures for the reliable assay of each of the enzymatic steps in crude extracts were devised. Crude extracts of the prototrophic haploid stock catalyzed all three enzymatic steps. Examination of a series of leucine auxotrophs permitted a correlation between the three enzymatic steps and the genetic lesions affecting 10 different loci. This examination revealed that a single locus (*le-6*) affected primarily α -isopropylmalate synthetase, the first step in the pathway. Lesions in six loci (*le-1*, *le-4*, *le-5*, *le-7*, *le-8*, and *le-10*) lead primarily to a deficiency in the activity of the second enzyme in the pathway, α -isopropylmalate isomerase. Stocks with lesions in three loci (*le-2*, *le-3*, and *le-9*) were primarily blocked in the third step of the pathway, catalyzed by β -isopropylmalate dehydrogenase. The results with the mutants provide strong evidence that the pathway for leucine biosynthesis proposed by Strassman and his colleagues is the sole significant pathway in yeast.

The biochemical steps in the biosynthesis of leucine have been clearly demonstrated in *Salmonella typhimurium*, *Neurospora crassa*, and in yeast (2, 6, 10, 19). The enzymatic steps in the pathway are exactly those postulated in 1956 by Strassman et al. (20):

Mutant methodology employed in the study with *Salmonella* and *Neurospora* demonstrated that specific enzymes are responsible for catalyzing reactions 1, 2, and 3. No mutants have yet been isolated which are completely blocked in reaction 4, and it has been inferred that more than one enzyme can catalyze that reaction. Because a study of yeast leucine auxotrophs had not yet been made, a collaborative study has been undertaken to correlate the genetic lesions with biochemical blocks in the leucine auxotrophs in the

Lindegren collection. From the outset, it had been anticipated that the study would be of interest, since 10 genetic loci leading to a leucine requirement had been observed in the genetic studies (G. Lindgren, *unpublished observations*) in contrast to the four lesions described for *Neurospora* (7) and *Salmonella* (16). The results of this collaborative study are reported here.

MATERIALS AND METHODS

Organisms. The wild-type organism was a prototrophic haploid yeast culture No. 60615 (alpha mating type) from the Lindgren breeding stock of *Saccharomyces* sp. (This stock was synthesized from six different species of *Saccharomyces* and may not properly be given a specific designation.)

The stock carries at least 14 mapped chromosomes, each with a differentiable centromere and several chromosomal segments, the centromeres of which have not yet been identified. The leucineless mutants were induced by treatment of the wild type with ethyl methanesulfonate (12). Ten different, non-identical, nonallelic genes were identified both by genetic experiments using tetrad analysis and by complementation. Many of the nonidentical genes were specifically located by linkage data and are on different chromosomes, namely, *le-1* is on chromosome VII, *le-2* on chromosome III, *le-3* and *le-4* on differ-

¹ Present address: Biological Research Laboratory, Southern Illinois University, Carbondale 62901.

ent segments, *le-6* on chromosome XII. The loci in classes 5, 7, 8, 9, and 10 have not yet been located.

Of the 29 leucineless mutants obtained, 14 were *le-1*, six *le-2*, one *le-3*, one *le-4*, one *le-5*, two *le-6*, one *le-7*, one *le-8*, one *le-9*, and one *le-10*.

Media. The basal medium in this study was modified from that of Halvorson (9) and contained (in 1 liter): ammonium sulfate, 4 g; dipotassium phosphate, 8.7 g; succinic acid, 5.8 g; calcium chloride, 0.3 g; magnesium sulfate hexahydrate, 0.5 g; trace element solution, 1.0 ml; vitamin mixture, 1.0 ml (added sterilely after autoclaving); and glucose, 10 g (added sterilely after autoclaving). The trace element solution was that of Lucas et al. (15) and contained 50 mg each of MnSO_4 , ZnSO_4 , and CuSO_4 . The vitamin mixture was also that of Lucas et al. (15) and contained (in 100 ml): biotin, 0.1 mg; calcium pantothenate, 30 mg; folic acid, 30 mg; and 330 mg each of inositol, pyridoxine, nicotinic acid, *p*-aminobenzoic acid, and thiamine. The vitamin mixture was sterilized separately at 10 psi for 5 to 10 min and stored in a frozen state in amber-colored bottles. Other supplements were employed as indicated. Stock cultures were stored on slants made from the basal medium supplemented with 2% agar (Difco), 0.5% tryptone (Difco), and 0.2% yeast extract (Difco).

Growth of cultures. For the growth of cells used to prepare extracts, batch cultures were employed. The cells were inoculated into 1 liter of the minimal medium supplemented, where indicated, with 2×10^{-3} M L-leucine and other growth factors in 2-liter Erlenmeyer flasks and incubated in a New Brunswick incubator-gyrotory shaker at 33 C. The inoculum was grown overnight in the same medium which, in turn, had been inoculated from a stock slant. Growth was measured turbidimetrically by using a Klett-Summerson colorimeter with a blue no. 42 filter by withdrawing 3-ml samples from the flasks aseptically at appropriate intervals.

Preparation of extracts. The organisms were harvested by centrifuging the cells in 250-ml cups in a Sorvall RC-2 centrifuge at 7,000 rev/min for 10 min. The pellet was washed once more with 0.01 M potassium phosphate buffer (pH 6.5) containing 2×10^{-3} M leucine. The washed pellets were resuspended in 100th the original volume of the same buffer, combined in weighed 50-ml Nalgene centrifuge tubes, and centrifuged at 15,000 rev/min for 15 min. The pellet thus collected was frozen for a short time to facilitate removal of the pellet quantitatively.

Usually, 1 g (wet weight) of cells was ground to a fine paste with 2 g of alumina in a precooled mortar and pestle. It was further ground with an additional 1 g of alumina for 5 min, and the paste was extracted with 0.05 M potassium phosphate buffer with 2×10^{-3} M leucine, using small amounts of the buffer. The paste was centrifuged at 15,000 rev/min for 15 min. The turbid, yellowish supernatant fluid was filtered through paper to yield a crude extract which was clear. Dialyzed crude extracts were prepared by dialyzing 2 ml of the extract in viscose tubing against 300 ml of 0.01 M potassium phosphate buffer (pH 6.5) containing 2×10^{-3} M leucine for 1 hr at 4 C with stirring.

Chemicals. α -Ketoisovaleric acid and α -ketoiso-

caproic acids were purchased from Sigma Chemical Co., St. Louis, Mo. The α -ketoisovaleric was neutralized with sodium carbonate and stored in the frozen state. Chromato pure lithium salt of coenzyme A (CoA) was purchased from P-L Company, Milwaukee, Wis. Acetyl CoA was made according to the method of Stadtman (18), and the concentration was checked according to the assay of Lipmann and Tuttle (13). α -Isopropylmalate (α -IPM) and β -isopropylmalate (β -IPM), isolated from the growth medium of *N. crassa* leucine mutant 33757, were gifts from Joseph Calvo and Samson Gross. Alcoa alumina (A-305) was purchased from Aluminum Carbide Company of America, Bauxite, Ark. All other chemicals were of reagent grade.

Enzyme assays. The α -IPM synthetase activity was assayed by a modification of the method of Burns et al. (1). The reaction mixture contained (in 0.5 ml): sodium α -ketoisovalerate, 5 μ moles; acetyl CoA, 0.5 μ moles; tris(hydroxymethyl)aminomethane (Tris)-chloride (pH 8.5), 50 μ moles; potassium chloride, 35 μ moles; and dialyzed crude extract (except where indicated otherwise). After 8 min at 30 C, the reaction was stopped by adding 3.0 ml of 0.2% metaphosphoric acid in saturated sodium chloride. Acetyl CoA cleavage was determined by using the nitroprusside assay of Grunert and Phillips (8) for free-SH groups. In rapid sequence, 0.5 ml of 2% sodium nitroprusside and 0.5 ml of a solution containing 1.5 M sodium carbonate and 67 mM sodium cyanide were added to the reaction mixture. The color was read immediately in a Klett-Summerson colorimeter with a green no. 54 filter. The nitroprusside assay was standardized with CoA. A reaction mixture from which α -ketoisovalerate had been omitted served to correct for -SH compounds present in the crude extract and for the acetyl CoA cleavage due to other activities in the crude extract. In routine assays of extracts for α -IPM synthetase activity, three concentrations of extract were employed to assure that the activities observed were in the linear range of the assay.

The activity of α -IPM isomerase was determined by following the increase in optical density at 235 nm due to the formation of dimethylcitrateconate (DMC) in a Gilford model 2000 recording spectrophotometer system, as described by Gross et al. (6). (At this wavelength, the extinction of a 1 mM solution is 4.53.) The reaction mixture contained (in 0.5 ml): potassium phosphate (pH 7.0), 40 μ moles; β -IPM, 1 μ mole; and crude extract. Substrate was omitted from the control tube. Routinely, two different extract concentrations were tested. The reaction was followed for at least 5 min at 30 C.

Because the crude extract contained very high reduced nicotinamide adenine dinucleotide (NADH) oxidase activity, the activity of β -IPM dehydrogenase was determined by measuring the amount of ketoacid formed from β -IPM, as described by Burns et al. (2). The reaction mixture contained (in 1 ml): Tris-chloride (pH 8.0), 300 μ moles; manganese sulfate, 0.5 μ moles; potassium chloride, 50 μ moles; nicotinamide adenine dinucleotide (NAD), 1 μ mole; β -IPM, 0.5 μ moles; and crude extract. A complication noted in certain extracts was due to an NADH-linked α -keto acid reductase activity. To minimize the significance

of this activity, short incubation times were employed (usually 1 and 2 min). In addition, three extract levels were tested to assure that the activities were determined in the linear range of the assay. The keto acid formed was determined on the entire reaction mixture by a modified Friedemann and Haugen (4) assay by adding 3.0 ml of a 0.025% solution of 2,4-dinitrophenylhydrazine in 0.5 N HCl. After 15 min at room temperature, 1.0 ml of 40% (w/v) potassium hydroxide was added, and the color was immediately determined in a Klett-Summerson colorimeter with a green no. 54 filter.

Protein concentration was determined by the method of Lowry et al. (14).

RESULTS

Assay of α -IPM synthetase activity in wild-type extracts. When crude extracts of yeast were examined for α -IPM synthetase activity by the procedure that had proven useful with bacterial extracts, evidence for the reaction was readily demonstrable. Furthermore, when appropriate enzyme levels were employed, the reaction was linear with time (Fig. 1A). However, with some extracts the activity was not linearly related to extract concentration unless the extracts were dialyzed or treated with Sephadex G-25. Dialysis removed from such an extract some factor which markedly stimulated the α -ketoisovalerate-dependent cleavage of acetyl CoA (Fig. 1B). Another observation that led to the impression that the stimulation might be specific for the α -IPM synthetase was that the stimulation, like activity itself (17), was inhibited by leucine.

Since it would be of interest to know the

identity of the stimulatory factor, an effort was made to identify it in boiled extracts. Boiled extracts did, in fact, stimulate, as did the Dowex-50-adsorbed and ammonia-eluted fraction. A synthetic mixture of 18 amino acids was tested; of these, it appeared that aspartate alone was stimulatory. Since aspartate did not stimulate acetyl CoA cleavage in the absence of α -ketoisovalerate, it appeared that the stimulation was due to the formation of oxaloacetate in a transamination reaction and that the oxaloacetate so formed served as substrate for the more potent citrate-condensing enzyme. Subsequent analysis revealed that the inhibition by leucine was not specific, but that certain other amino donors, such as valine (Table 1), decreased the stimulation, apparently by altering the ratio of the transamination reactants away from oxaloacetate.

Dialysis for a short period against buffer supplemented with leucine did not appear to decrease the activity since the curve obtained with the undialyzed extract deviates very little from the curve obtained using dialyzed extract at low extract concentrations (Fig. 1B). Therefore, the routine adopted was to dialyze one portion of the extract for 1 hr immediately after preparation. During this period, the assays of the other activities were performed.

The buffer used for suspending the cells during disruption was supplemented with 2×10^{-3} M L-leucine. It was found early in the study that inclusion of leucine in the disruption buffer resulted in extracts containing greater α -IPM synthetase activity (Table 2). As we will show

FIG. 1. Effect of time and protein concentration on the formation of α -isopropylmalate by yeast extracts. (A) Amount of product formed by using dialyzed crude extract of yeast containing 0.94 mg of protein in the standard assay system. (B) $\square$, Product formed after 8 min by an undialyzed crude yeast extract; $\circ$, product formed after 8 min by the same crude extract after dialysis.

later (17), leucine is an inhibitor of the enzyme, and it might not be surprising if it were also a stabilizer of the enzyme. Therefore, one possibility was that the greater yield of enzyme activity was a consequence of greater stability in those extracts containing leucine.

To test this possibility, the stability in ice of an extract prepared in the usual way was compared with that of an extract prepared with buffer from which leucine was omitted. The loss, after 3.5 hr, was the same in the two preparations (Table 2).

Assay of α -IPM isomerase activity in wild-type extracts. There was proportionality between the amount of isomerase activity and extract concentration over the range of 6 to 25 μ g of yeast protein (Fig. 2). With the concentrations of β -IPM employed, the reaction rate remained constant over the period of measurement. All the activity measurements reported in this paper were obtained by using β -IPM. These activities were about 3.5 times those obtained with DMC as substrate.

Like the isomerase activities in *Neurospora* and *Salmonella* (6), no metal or other cofactor requirement could be demonstrated. Reduced glutathione or ferric ions did not stimulate but, rather, were slightly inhibitory when added to the reaction mixture. Compared to the other systems, a substrate concentration about 10 times higher had to be employed to obtain a valid isomerase

FIG. 2. Effect of protein concentration on the formation of dimethylcitrate by a crude extract of yeast.

activity in yeast extracts. The isomerase activity was also quite unstable, especially in diluted extracts. Although the routine assay required dilution of the extract, it was necessary to prepare the dilution immediately before the assay. To minimize the effect of this instability, the enzyme was assayed within 20 min after preparation of the extract.

Assay of β -IPM dehydrogenase in wild-type extracts. Although the dehydrogenase was also unstable under the conditions that were employed, the activity was linearly related to time and extract concentration (Fig. 3). Again, by performing the assay immediately after preparation of the extract, it was found that the effect of the instability of the activity was minimized. Attempts to stabilize the enzyme by using α -IPM, β -IPM, and α -ketoisocaproate or pyridoxal phosphate were all unsuccessful.

Another difficulty in the determination of dehydrogenase activity, which was sometimes encountered in extracts, was an α -keto acid reductase activity in which the α -keto acid product was presumably reduced by the other product of the reaction, NADH. Attempts to take advantage of the observation of Ciaccio (3), that the activity of lactic dehydrogenase is inhibited by bisulfite or by 3-acetyl pyridine adenine dinucleotide, were in vain. This difficulty might also have been bypassed if analogues of NAD could have been employed as hydrogen acceptor in the assay. However, those that were available for test were inactive.

Biochemical blocks in various leucine auxotrophs of yeast. For a preliminary screening of the enzymatic deficiency in mutant strains, batch cultures were employed. Thus, if leucine were a

TABLE 1. Effect of aspartate on α -IPM synthetase activity

Additions to complete system	Specific activity ^a
None.....	0.013
L-Aspartate, 4×10^{-4} M.....	0.042
L-Aspartate, 4×10^{-4} M plus L-Valine, 10^{-2} M.....	0.013
L-Valine, 10^{-2} M.....	0.014

^a Expressed as micromole of CoA formed per milligram of protein per minute.

TABLE 2. Effect of L-leucine in the buffer used to suspend the cells for disruption

Suspending buffer	Specific activity ^a	
	Immediately after prepn	After 3.5 hr
0.05 M potassium phosphate (pH 6.5).....	0.010	0.006
Same, with 2×10^{-3} M L-leucine.....	0.015	0.009

^a Expressed as micromole of CoA formed per milligram of protein per minute.

FIG. 3. Effect of time and protein concentration on the formation of α -ketoisocaproate from β -isopropylmalate by a crude extract of yeast. The extract contained 24 mg of protein per ml. Symbols: $\circ$, 1 min; $\square$, 2 min.

repressor of the enzymes in the pathway to leucine, the enzymes would be at a repressed level. Table 3 shows the results of testing one arbitrarily selected representative of each of the 10 genetic classes of leucine auxotrophs. Only one class, *le-6*, appeared to be deficient in the first enzyme in the pathway α -IPM synthetase. The very low levels of the second and third enzymes is of interest in view of the observation in *N. crassa* that the appearance of these enzymes is dependent upon the presence of a functional synthetase (5). However, in view of the variation in levels of the enzymes in the other mutants, no conclusion can be drawn from this observation alone.

Lesions in 6 of the 10 genetic loci (*le-1*, *le-4*, *le-5*, *le-7*, *le-8*, and *le-10*) resulted in failure only to produce detectable levels of α -IPM isomerase (Table 3). The remaining three loci (*le-2*, *le-3*, and *le-9*) appeared to be concerned with the dehydrogenase, although, in the mutant with the *le-9* lesion, isomerase was undetectable.

Several of the mutant strains, although absolutely blocked in only one step, had low levels of one or both of the remaining two enzymes (Table 3). The results with these strains were reproducible, but the pattern exhibited is probably not lesion-specific. As will be shown in the following report (17), different strains within one class vary in the levels of the two remaining enzymes. For example, in the only other *le-4* mutant that was tested, none of the three enzymes could be detected.

TABLE 3. Leucine biosynthetic enzymes in a series of yeast auxotrophs

Strain	Locus affected	Specific activity ^a		
		Synthetase	Isomerase	Dehydrogenase
60615	None	0.059	0.044	0.024
Q 178	<i>le 1</i>	0.020	0.000	0.024
Q 174	<i>le 2</i>	0.044	0.063	0.000
12524B	<i>le 3</i>	0.008	0.007	0.000
65279	<i>le 4</i>	0.015	0.000	0.011
Q 121	<i>le 5</i>	0.013	0.000	0.010
70458	<i>le 6</i>	0.000	Trace	0.008
74394	<i>le 7</i>	0.017	0.000	0.018
75290	<i>le 8</i>	0.031	0.000	0.002
Q 1087	<i>le 9</i>	0.010	Trace	0.000
75315	<i>le 10</i>	0.003	0.000	0.024

^a Minimal detectable levels of activity in these extracts were: for the synthetase, 0.0023; for the isomerase, 0.0030; for the dehydrogenase, 0.0020. Expressed as micromole of product formed per milligram of protein per minute.

DISCUSSION

With the correlation between the biochemical blocks and genetic lesions affecting leucine biosynthesis in the Lindgren yeast stock described in this report, it was possible to establish, by mutant methodology, the obligatory nature of the enzymatic steps first proposed by Strassman et al. (20) on the basis of isotope incorporation studies. That the mutants did provide proof of that pathway was not surprising in view of the earlier studies with *Salmonella* and *Neurospora* (2, 6, 10) and the demonstration by Strassman and co-workers of the proposed steps (19) in extracts of bakers' yeast. More surprising was the genetic finding that 10 different loci appeared to affect a pathway of only three enzymes (or four enzymes if the conversion of α -ketoisocaproate to leucine had been catalyzed by a specific transaminase).

While it is not yet possible to state that each of the 10 loci is a structural gene for a polypeptide chain in one of the leucine-forming enzymes, it is noteworthy that the lesion in each of the 10 loci was found to affect primarily one of the three enzymes. Thus, none appeared to have a regulatory function affecting all three enzymes in an all-or-none manner. It may be that some of the loci have a regulatory function affecting primarily one enzyme, while affecting only secondarily the other two. However, it is with some skepticism that one asks whether each of the six loci affecting the isomerase could correspond to a distinct subunit of the enzyme. A more attractive

possibility is that leucine biosynthesis occurs in a distinct organelle, much like that which Wagner (Kiritani et al., 11) has postulated for the isoleucine- and valine-forming enzymes of *Neurospora*. It is possible that noncatalytic subunits have a "carrier" function and that some secondarily have acquired the function of stabilizing the isomerase or the dehydrogenase. No evidence for such a possibility has been found. Attempts are currently being made to purify the isomerase in order to determine the number of subunits in that enzyme.

ACKNOWLEDGMENT

This work was supported by Public Health Service grants GM 12522 and GM 14474 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Burns, R. O., J. Calvo, P. Margolin, and H. E. Umbarger. 1966. Expression of the leucine operon. *J. Bacteriol.* **91**:1570-1576.
2. Burns, R. O., H. E. Umbarger, and S. R. Gross. 1963. The biosynthesis of leucine. III. The conversion of α -hydroxy- β -carboxyisocaproate to α -ketoisocaproate. *Biochemistry* **2**:1053-1058.
3. Ciaccio, E. I. 1965. The inhibition of lactate dehydrogenase by 3-acetylpyridine adenine dinucleotide and bisulfite. *J. Biol. Chem.* **241**:1581-1586.
4. Friedemann, T. E., and G. E. Haugen. 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. *J. Biol. Chem.* **147**:415-442.
5. Gross, S. R. 1965. The regulation of synthesis of leucine biosynthetic enzymes in *Neurospora*. *Proc. Natl. Acad. Sci. U.S.A.* **54**:1538-1546.
6. Gross, S. R., R. O. Burns, and H. E. Umbarger. 1963. The biosynthesis of leucine. II. The enzymic isomerization of β -carboxy- β -hydroxyisocaproate and α -hydroxy- β -carboxyisocaproate. *Biochemistry* **2**:1046-1052.
7. Gross, S. R., and H. S. Gross. 1961. Some features of complementation at the leucine-4 locus of *Neurospora*. *Genetics* **46**:868-869.
8. Grunert, R. R., and P. H. Phillips. 1951. A modification of the nitroprusside method of analysis for glutathione. *Arch. Biochem.* **30**:217-225.
9. Halvorson, H. O. 1958. Studies on protein and nucleic acid turnover in growing cultures of yeast. *Biochim. Biophys. Acta* **27**:267-276.
10. Jungwirth, C., S. R. Gross, P. Margolin, and H. E. Umbarger. 1963. The biosynthesis of leucine. I. The accumulation of β -carboxy- β -hydroxyisocaproate by leucine auxotrophs of *Salmonella typhimurium* and *Neurospora crassa*. *Biochemistry* **2**:1-6.
11. Kiritani, K., S. Narise, A. Bergquist, and R. P. Wagner. 1965. The overall *in vitro* synthesis of valine from pyruvate by *Neurospora* homogenates. *Biochim. Biophys. Acta* **100**:432-443.
12. Lindegren, G., Y. L. Hwang, Y. Oshima, and C. C. Lindegren. 1965. Genetical mutants induced by ethyl methanesulfonate in *Saccharomyces*. *Can. J. Genet. Cytol.* **7**:491-499.
13. Lipmann, R., and L. C. Tuttle. 1945. A specific micromethod for the determination of acyl phosphates. *J. Biol. Chem.* **159**:21-28.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
15. Lucas, J. M., A. H. W. M. Schuurs, and M. V. Simpson. 1964. A cell-free amino acid-incorporating system from *Saccharomyces cerevisiae*. Variation in ribosomal activity and in RNA synthesis during logarithmic growth. *Biochemistry* **3**:959-967.
16. Margolin, P. 1962. Genetic fine structure of the leucine operon in *Salmonella*. *Genetics* **48**:441-457.
17. Satyanarayana, T., H. E. Umbarger, and G. Lindegren. 1968. Biosynthesis of branched-chain amino acids in yeast: regulation of leucine biosynthesis in prototrophic and leucine auxotrophic strains. *J. Bacteriol.* **95**:2018-2024.
18. Stadtman, E. R. 1953. The coenzyme A transphorase system in *Clostridium kluyveri*. *J. Biol. Chem.* **203**:501-512.
19. Strassman, M., and L. N. Ceci. 1963. Enzymatic formation of α -isopropylmalic acid, an intermediate in leucine biosynthesis. *J. Biol. Chem.* **238**:2445-2452.
20. Strassman, M. L., A. Locke, A. J. Thomas, and S. Weinhouse. 1956. A study of leucine biosynthesis in *Torulopsis utilis*. *J. Am. Chem. Soc.* **78**:1599-1602.