EXPERIENTIA

Vol. XII - Fasc. 7 | Pag. 245–284 | 15. VII. 1956

Biochemical Factors Affecting Symbiosis Among Bacteria

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The symbiotic interrelationships of micro-organisms among microbes or between microbes and higher organisms, are of fundamental importance in nature. Even a few classic examples are sufficient to indicate the great significance of the symbiosis phenomenon in biology and human ecology. Among the most important of these are symbiosis between root-nodule bacteria and leguminous plants, mycorrhiza formation between certain fungi and higher plants and lichen formation between algae and fungi. In addition, mention may be made of the cooperative associations of cellulose-decomposing fungi (or bacteria) and nitrogen-fixing bacteria in which, however, the relationships between the different organisms are not so strictly symbiotic as in the former cases.

Despite the fact that symbiotic and other associative relationships of microorganisms play an important role in nature and also in many fields of industrial microbiology, the functional mechanisms in symbiotic systems have, from a biochemical point of view, been comparatively little explored. (It may be stated that only symbiotic nitrogen fixation has been extensively studied by biochemists, e.g. by VIRTANEN and co-workers.) Consequently, our present knowledge of the metabolic pathways and chemical steps involved in the symbiotic interrelationships existing between different organisms is also, generally speaking, very meagre.

In particular, the lactic acid bacteria have a great ability to enter into associative relationships in mixed cultures. Their significance in the dairy industry is a well known example of their great importance. However, the associative, and especially symbiotic, relationships of lactic acid bacteria have been very little explored, and the biochemical nature of the associative growth is mostly not definitely known. On the other hand our knowledge concerning the nutritional requirements of these bacteria has increased extensively during the recent years (e.g. reviewed by SNELL[2] and RITTER[3]), and this affords new possibilities also for the study of the biochemistry of associative relationships between lactic acid bacteria. The recent investigation has therefore been made with these bacteria with the special aim to obtain information concerning the biochemical factors underlying associative growth. The purpose of this article is to summarize the results of these experiments and it may conveniently be divided into the following sections: (1) Symbiosis between different species of lactic acid bacteria. (2) The symbiotic technique in the study of synthetic pathways of growth factors. (3) Conclusion.

Symbiosis between different species of lactic acid bacteria

During the course of investigation of factors influencing the associative growth of lactic acid bacteria it was found by the author in 1952 that close symbiotic interrelationships may exist between different species of these organisms[4]. When certain vitamin(s) and amino acid(s) essential for the growth of two strains were omitted from the otherwise complete synthetic medium (described by HENDERSON and SNELL[5]), both organisms were able to grow in symbiosis despite this lack, each producing the vitamins and amino acids needed by the other. On the basis of microbiological determinations made from the culture filtrates, it was concluded that the growth factors permitting symbiotic growth must be those vitamins and amino acids which are required for the growth of the separate symbionts. Figure 1 shows a typical example of these experiments. Two lactic acid bacteria *Streptococcus faecalis R* and *Lactobacillus arabinosus 17–5* did not grow alone, but were able to grow together in a medium lacking folic acid (pteroylglutamic acid) and phenylalanine, although folic acid is required by *Str. faecalis R* and phenylalanine by *Lb. arabinosus 17–5*.

Symbiosis was likewise exhibited by the following pairs of lactic acid bacteria in synthetic media lacking other essential growth factors[6]. *Lb. arabinosus 17–5*

[1] Laboratory of Valio, Biochemical Institute, Helsinki, Finland.

[2] E. E. SNELL, in: *Bacterial Physiology*, edited by C. H. WERKMAN and P. W. WILSON (Acad. Press, New York 1951), p. 214. – E. E. SNELL, Bacteriol. Rev. *16*, 227 (1952).

[3] W. RITTER, VIth Intern. Congress for Microbiology, Roma, Symposium Nutrition and Growth Factors 1954, p. 157.

[4] V. NURMIKKO, Acta chem. Scand. *6*, 1258 (1952).

[5] L. M. HENDERSON and E. E. SNELL, J. biol. Chem. *172*, 15 (1948).

[6] V. NURMIKKO, *Symbiosis Experiments Concerning the Production and Biosynthesis of Certain Amino Acids and Vitamins in Associations of Lactic Acid Bacteria*, Ann. Acad. Sci. Fennicae [A II] *54*, 1–58 (1954).

(a phenylalanine-requiring strain) and *Str. faecalis R* (a folic acid-, threonine-, histidine-, and serine- or glycine-requiring strain) can grow in symbiosis when (*a*) phenylalanine, threonine and folic acid, or (*b*) phenylalanine and histidine, or (*c*) phenylalanine, serine and glycine were omitted from the synthetic medium. *Leuconostoc mesenteroides P—60* (a proline-, phenylalanine-, and glycine-requiring strain) and *Str. faecalis R* (a folic acid-requiring strain) were able to grow in symbiosis

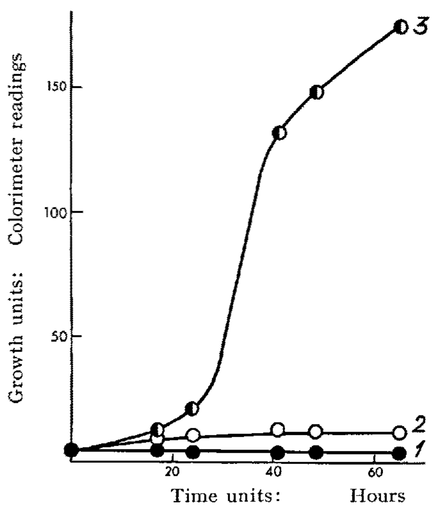

Fig. 1.—Growth of *Lactobacillus arabinosus 17—5* and *Streptococcus faecalis R* in symbiosis. Synthetic basal medium without phenylalanine and folic acid (pteroylglutamic acid). Curve 1: *Str. faecalis R* (folic acid-requiring strain). Curve 2: *Lb. arabinosus 17—5* (phenylalanine-requiring strain). Curve 3: *Lb. arabinosus 17—5* and *Str. faecalis R* together.

in media lacking (*a*) proline and folic acid or (*b*) proline, phenylalanine, glycine, and folic acid. Further *Lb. fermenti—36* (a phenylalanine-requiring strain) and *Str. faecalis R* (a folic acid-requiring strain) were able to grow in symbiosis in a medium lacking folic acid and phenylalanine, and likewise *Lb. leichmannii* (ATCC 4797) (vitamin B_{12}-requiring strain) and *Leuconostoc citrovorum* (ATCC 8081) (folinic acid-requiring strain) in a medium (described by STEELE *et al.*[7]) from which vitamin B_{12} and folinic acid were omitted[8]. A similar symbiosis experiment as the lastmentioned was made recently also by DOCTOR and COUCH[9].

On the basis of these symbiosis experiments it was concluded that the following growth factors (or in certain cases probably compounds possessing their activity) were excreted into the growth medium by the lactic acid bacteria used: phenylalanine, proline, threonine, histidine, glycine, serine, folic acid, folinic acid, and vitamin B_{12}. The amounts of some amino acids and vitamins were also determined using the microbiological assay method.

Similar interrelationships were observed in experiments in which different species of lactic acid bacteria were separated from each other by a dialyzing membrane[10]. Using a dialysis cell with several compartments especially designed for these experiments it was found that different species can grow not only in pairs in symbiosis, but also in associations of more than two different species. Figure 2 shows a symbiosis experiment made with six species of lactic acid bacteria. Among these bacteria only *Str. lactis* (ATCC 7963) was able to grow alone in a medium (described by ANDERSON and ELLIKER[11]) lacking folic acid, folinic acid, and phenylalanine. Of these growth factors folic acid was required by *Str. faecalis R*, folinic acid by *Ln. citrovorum* (ATCC 8081), and phenylalanine by *Lb. arabinosus 17—5*, *Lb. fermenti—36*, and *Ln. mesenteroides P—60*. Although these essential growth factors were omitted from the medium all six strains were able to grow despite this lack when inoculated into separate compartments of the dialysis cell. The growth in the compartments was found to be due to the ability of the different species to synthesize and excrete into the medium vitamins and amino acids required by the strain of the adjoining compartment. The growth factors produced in each compartment then passed

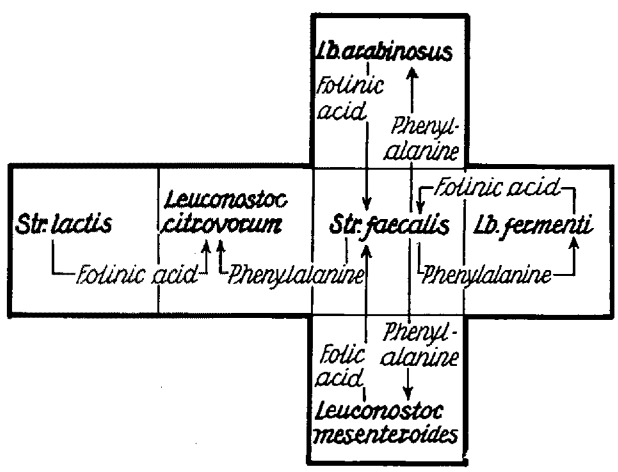

Fig. 2.—Symbiosis between six different species of lactic acid bacteria in a dialysis cell with six compartments.

across the dialyzing membrane to each adjoining compartment, permitting symbiotic growth in the dialysis cell.

The symbiotic technique in the study of synthetic pathways of growth factors

The symbiotic technique is based on the fact that different species and strains of lactic acid bacteria are

[7] B. F. STEELE, H. E. SAUBERLICH, M. S. REYNOLDS, and C. A. BAUMAN, J. biol. Chem. *177*, 533 (1949).

[8] V. NURMIKKO, VI[th] Intern. Congress for Microbiology, Roma, Symposium Nutrition and Growth Factors 1953, p. 97.

[9] V. M. DOCTOR and J. R. COUCH, Arch. Biochem. Biophys. *51*, 530 (1954).

[10] V. NURMIKKO, Acta chem. Scand. *9*, 1317 (1955).

[11] A. W. ANDERSON and P. R. ELLIKER, J. Dairy Sci. *36*, 161 (1953).

able to carry out defined steps in the biosynthesis of certain growth factors and that during growth they are also capable of excreting growth factors into the medium. By cultivating together two strains, each capable of carrying out only a limited part of the biosynthesis of a certain growth factor, it is possible to form a symbiotic system which may afford possibilities of investigating in detail the synthetic pathway of the growth factor concerned. The principles of this method may be illustrated by means of *the following scheme:*

Symbiotic system formed by two lactic acid bacteria

Organism	Enzyme systems catalyzing the following reaction sequences are present	Enzyme systems catalyzing the following reaction sequences are absent	Requires as essential growth factor	Growth factor excreted into the medium
1	$A \to B \to C \searrow P$	$C \to D \to E$	E	C
2	$C \to D \to E$, $\searrow P$	$A \to B \to C$	C	E

The symbiotic system shown above is formed by two organisms each able to carry out only a limited reaction sequence in the biosynthesis of a growth factor E. It depends on the deficient synthetic abilities of the two organisms that the growth factor E is required by one of these organisms (organism 1) and an intermediate product C by the other (organism 2). In addition to forming an intermediate stage in the synthesis of E, compound C also functions as a precursor in the synthesis of another compound, P.

When both essential growth factors C and E are omitted from the nutrient solution neither of the organisms is able to grow in this incomplete medium alone. However, when inoculated together, they can grow well. The symbiotic growth is due to the fact that organism 1 synthesizes and excretes into the medium the growth factor C required by organism 2, which then synthesizes the compound E, an essential growth factor for organism 1.

If, for instance, one wants to investigate possible intermediates existing in the reaction sequence $C \to D \to E$, not only compounds C and E but also compound D, which is supposed in this example to be the only known intermediate in this reaction sequence, are omitted from the nutrient solution. If the reaction(s) catalyzed by the growth factor E is (are) known, the endproduct(s) of this reaction can perhaps also be excluded from the medium, in order that the requirement of organism 1 for the growth factor E may increase. The exclusion of the intermediate D and the endproduct(s) of the reaction(s) catalyzed by the

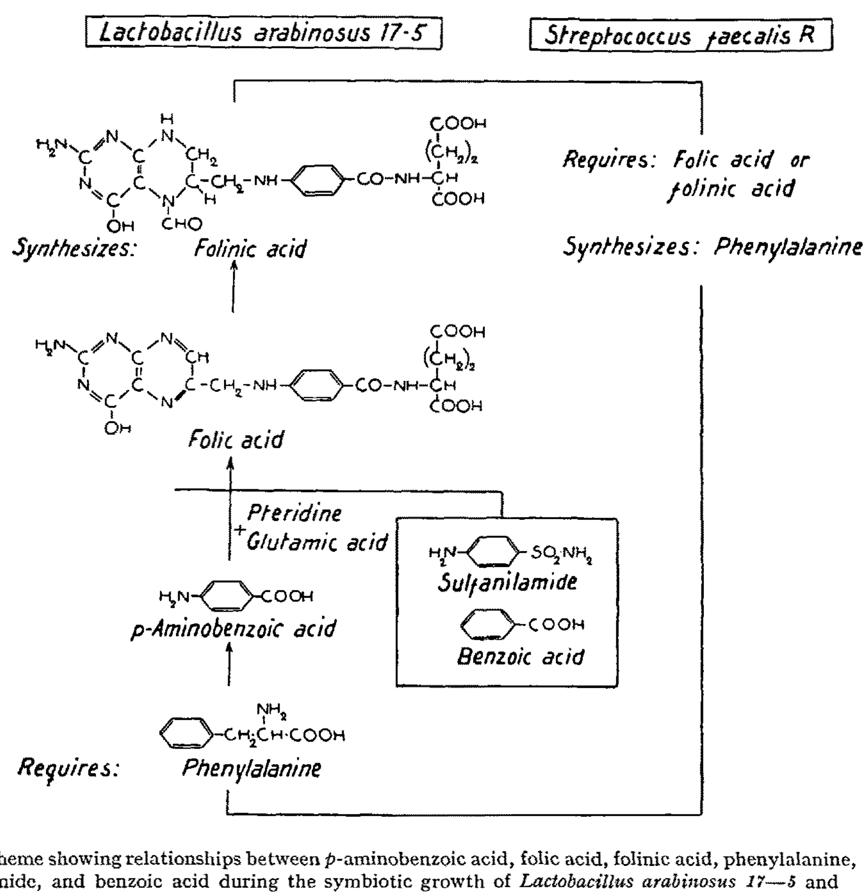

Fig. 3.—Scheme showing relationships between *p*-aminobenzoic acid, folic acid, folinic acid, phenylalanine, sulfanilamide, and benzoic acid during the symbiotic growth of *Lactobacillus arabinosus 17—5* and *Streptococcus faecalis R.*

growth factor E may result in a distinct retardation in the symbiotic growth. By investigating which of the compounds related to C, D, and E have the ability to restore the initial vigorous symbiotic growth it is possible to conclude which compounds may function as intermediates in the reaction sequence $C \rightarrow D \rightarrow E$. If those compounds which are able to replace D and E are denoted as d_1, d_2, d_3, and e_1, e_2, e_3 one gets the following reaction sequence as the synthetic pathway for the growth factor E from the precursor C:

$$C \rightarrow d_1 \rightarrow d_2 \rightarrow d_3 \rightarrow D \rightarrow e_1 \rightarrow e_2 \rightarrow e_3 \rightarrow E.$$

It should be especially noted that the essential growth factor C is a precursor for *two* different biosyntheses. It is this fact which makes it very difficult, and in some cases even impossible, to investigate the reaction $C \rightarrow E$ by using organism 1 alone. One should then *at the same time* have to examine which compounds belong to the reaction stages $C \rightarrow D$ and $C \rightarrow P$. If the compound C, for instance, is an amino acid required by organism 1 for its protein synthesis (P), the finding of suitable intermediate compounds for the reaction $C \rightarrow P$ presents extreme difficulties at the moment.

Using a symbiotic system formed by the species *Lb. arabinosus 17—5* and *Str. faecalis R*, the author has made an investigation into the biosynthesis of *p*-aminobenzoic acid[12]. It was found that the metabolic reaction sequences of these strains with respect to the growth factors phenylalanine, *p*-aminobenzoic acid, folic acid, and folinic acid were coupled together in the way shown in Figure 3. As appears from the scheme, *Str. faecalis R* was able to carry out only the first part of the biosynthesis of folinic acid, up to phenylalanine, and *Lb. arabinosus 17—5* only the latter part of this synthesis, i.e., the reaction phase phenylalanine → folinic acid.

To investigate with this symbiotic system the mechanism involved in the biosynthesis of *p*-aminobenzoic acid, the growth factors phenylalanine, *p*-aminobenzoic acid, folic acid, and folinic acid were omitted from the medium, a distinct retardation in the symbiotic growth resulting. By testing which of the compounds related to *p*-aminobenzoic acid and phenylalanine were able to restore the initial symbiotic growth, it was possible to discover some compounds which may constitute intermediates in the synthetic pathway of this vitamin. On the basis of the observations made it seems reasonable to postulate the following mechanism for the biosynthesis of *p*-aminobenzoic acid:

shikimic acid → phenylalanine → (intermediate: *p*-aminophenylpyruvic acid or *p*-aminophenylpropionic acid) → *p*-aminophenylacetic acid → *p*-aminobenzoic acid.

Moreover, the finding that phenylalanine has a powerful influence upon the production of folinic acid by *Str. lactis* (ATCC 7963) confirms the assumption that in lactic acid bacteria this amino acid functions as a precursor of *p*-aminobenzoic acid[13], a vitamin which is in itself an intermediate compound in the synthesis of folinic acid.

Conclusion

In the light of the experimental findings obtained in this investigation, it seems very probable that symbiosis among micro-organisms affords, from the biochemical point of view, a new and interesting object for research with possibilities of elucidating various metabolic processes in the living cell. Summarizing, it may be stated that this investigation showed that the different species of lactic bacteria growing in associations were capable of forming close symbiotic interrelationships in nutritionally deficient media. It was also found that the symbiosis phenomenon can be applied to the study of the mechanism of the biosynthesis of growth factors. Using this method, called the symbiotic technique, evidence has been obtained indicating that the amino acid phenylalanine may function as a precursor in the biosynthesis of the vitamin *p*-aminobenzoic acid. It should especially be noticed that in the experimental example given, the phenylalanine required by *Lb. arabinosus 17—5* is an essential precursor for two different syntheses, namely for that mentioned above, and in addition for protein synthesis. This being the case, the investigation of the mechanism of conversion of phenylalanine to *p*-aminobenzoic acid using *Lb. arabinosus 17—5* alone as a growing system would present great experimental difficulties. Also the fact, discovered in symbiosis experiments, that *p*-aminobenzoic acid is an essential vitamin for the growth of *Lb. arabinosus 17—5*, but only in the absence of phenylalanine, seems very difficult to establish using this organism alone. As these observations indicate, it is possible by means of the symbiotic technique to discover new growth factor requirements in bacteria, and also to investigate the biosynthetic pathways of growth factors where, perhaps, investigations using the classical pure culture methods would have given no result.

Finally, it ought to be emphasized that our knowledge of the living processes in the microbial cell is based almost entirely on the pure culture studies. However, pure cultures of a single micro-organism seldom exist in nature. On the contrary, microbes live in soils, water, alimentary tract of human beings and animals etc., in mixed populations with complex interrelationships. Consequently, in the pure culture the

[12] V. NURMIKKO, Acta chem. Scand. *7*, 942 (1953); Ann. Acad. Sci. Fennicae [A II] *54*, 1–58 (1954).

[13] V. NURMIKKO, Suomen Kemistilehti [B] *28*, 62 (1955).

metabolic functions of a certain microorganism may not be a true representation of its chemical activity in nature. Particularly therefore there is a need of investigating the biochemical mechanisms whereby the metabolic processes of associative organisms are coupled together. On the basis of the findings described in this paper it may be concluded that the symbiotic technique as opposed to the classical pure culture method is useful in the study of the biochemistry of microbial associations existing in different natural conditions.

Acknowledgements. I wish to express my gratitude to Professor ARTTURI I. VIRTANEN, Director of the Biochemical Institute, for valuable discussions and criticism. I also wish to thank Professor E. E. SNELL, University of Texas, for the strains of lactic acid bacteria, Dr. H. P. BROQUIST, American Cyanamid Company, New York, for the sample of leucovorin, and Dr. B. D. DAVIS, Cornell University Medical College, for the sample of shikimic acid.

Zusammenfassung

Die zusammenwirkenden, insbesondere symbiontischen Faktoren, die in einer Bakterienmischkultur zur Wirkung kommen, sind noch wenig bekannt. Der vorliegende Übersichtsartikel orientiert über die gegenseitige Förderung verschiedener Milchsäurebakterien in synthetischen Nährlösungen mit besonderer Berücksichtigung einiger biochemischer Faktoren. Bei allen Versuchen wurden bestimmte, für das Wachstum unentbehrliche Vitamine oder Aminosäuren aus den Nährlösungen weggelassen. Es ergab sich, dass verschiedene Milchsäurebakterienstämme in einer Nährlösung miteinander gedeihen können, in welcher der eine oder der andere Stamm für sich allein mangels lebenswichtiger Vitamine oder Aminosäuren nicht hätte gedeihen können. Einige Milchsäurebakterien sind somit zur Synthese der von anderen Organismen benötigten Vitamine und Aminosäuren befähigt. Beispiele: *Lactobacillus arabinosus 17–5* bildet die für *Streptococcus faecalis R* notwendige Folsäure (oder Folinsäure) und umgekehrt *Str. faecalis R* das für *Lb. arabinosus 17–5* unentbehrliche Phenylalanin (Abb. 1).

Auf Grund dieser symbiontischen Beziehungen zwischen verschiedenen Milchsäurebakterienstämmen wurde eine Methode, die sogenannte Symbiose-Technik, für die Untersuchungen der Biosynthese der verschiedenen Wachstumsfaktoren entwickelt. Die theoretische Grundlage dieser Methode ist vom Verfasser ausgearbeitet worden. Mit Hilfe dieser Methode wurden Untersuchungen über die Biosynthese von *p*-Aminobenzoesäure ausgeführt. Die Versuche zeigen, dass die Aminosäure Phenylalanin bei Milchsäurebakterien als Vitamin-Vorstufe auftritt.

Die Ergebnisse lassen vermuten, dass die erwähnten zusammenwirkenden symbiontischen Wachstumsfaktoren (Aminosäuren und Vitamine) bei der Entwicklung von Bakterienmischkulturen in der Natur eine wichtige Rolle spielen. Der Verfasser hebt besonders hervor, dass das Studium der Symbiose in der Natur mit Hilfe dieser neuesten Methode sehr interessant wäre. Es lässt sich denken, dass zum Beispiel bei diesem Vorgehen neue biochemische Prozesse und Wachstumsfaktoren gefunden werden, die mit Hilfe der klassischen Reinkulturmethoden nur schwierig festzustellen wären.

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Rauwolfia Alkaloids XXVII
The Conversion of 3-Isoreserpine to Reserpine

The synthesis of *d,l*-3-isoreserpine recently reported by WOODWARD[1] gives added import to the conversion of 3-isoreserpine to reserpine for which we wish to record two methods. The epimerization of reserpine and its derivatives at C-3 was previously stated to proceed to completion under the reaction conditions employed as far as could be measured[2]. This conclusion was based on the failure to isolate unepimerized reserpine from the reaction mixture. Mindful of the limitations of this means of assaying the completeness of the reaction, we applied the more precise method of paper chromatographic analysis to the problem. In the system benzene-cyclohexane (1:1) as the mobile phase on formamide-methanol (7:3) impregnated Whatman No. 1 paper, reserpine and 3-isoreserpine had R_f values of 0·32 and 0·82 respectively. This is a slight modification of the system already described for the paper chromatography of *Rauwolfia* alkaloids[3].

A study of the isomerization of reserpine in various new media led to the observation that in refluxing acetic acid a considerable amount of reserpine remains unepimerized. 3-Isoreserpine, prepared as already described[4]

[1] R. B. WOODWARD, Nichols Award Address, March 16, 1956. New York, N. Y.

[2] H. B. MACPHILLAMY, L. DORFMAN, C. F. HUEBNER, E. SCHLITTLER, and A. F. ST. ANDRÉ, J. Amer. chem. Soc. *77*, 1071 (1955). – H. B. MACPHILLAMY, C. F. HUEBNER, E. SCHLITTLER, A. F. ST. ANDRÉ, and P. R. ULSHAFER, J. Amer. chem. Soc. *77*, 4335 (1955).

[3] F. A. HOCHSTEIN, K. MURAI, and W. H. BOEGEMANN, J. Amer. chem. Soc. *77*, 3551 (1955). – A. F. ST. ANDRÉ, B. KORZUN, and F. WEINFELDT, J. org. Chem. *21*, in press (1956).

[4] H. B. MACPHILLAMY, C. F. HUEBNER, E. SCHLITTLER, A. F. ST. ANDRÉ, and P. R. ULSHAFER, J. Amer. chem. Soc. *77*, 4335 (1955).