THE BIOSYNTHESIS OF ^{13}C LABELED STARCH GRANULES AS A SOURCE OF UNIFORMLY LABELED ^{13}C D-GLUCOSE

J.R.Buchholz*, C.W.Christenson*, R.T.Eakin** and E.Fowler*

Received on February 8, 1973

SUMMARY

A potato plant has been grown in a closed chamber with CO_2 enriched to a level of 31 at. % in ^{13}C as the sole source of carbon. In 80 days the plant produced 1,684 g of tubers labeled with 29 at. % ^{13}C. Of the carbon fixed by the potato plant approximately 70% was found in the tubers while 30% was found in the tops and roots. Recovered starch granules constituted 10% of the fresh weight of these tubers. A 16% yield of glucose was obtained, based on all of the carbon dioxide used. Distribution of the ^{13}C label among the six species of carbon atoms in starch appears to be nearly uniform. The plant and tubers were indistinguishable from those grown on normal isotopic CO_2 indicating no gross isotope effect at this level of ^{13}C enrichment.

INTRODUCTION

Recent developments now make available unprecedented quantities of carbon highly enriched in the ^{13}C isotope at a fraction of the previous cost.[1,2] Chemical modifications and syntheses starting with carbon monoxide enriched in ^{13}C are being performed to provide compounds for use in the biological,

*Health Division, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico, United States of America.

**Visiting Staff Member, Los Alamos Scientific Laboratory, University of California, and Department of Chemistry, University of Texas, Austin, Texas, United States of America.

physical, and environmental sciences.[2] The nuclear spin and stability of the ^{13}C nucleus make this isotope useful as a tracer and in structural elucidations by magnetic resonance techniques.

Uniformly labeled D-glucose, which is readily obtained by biosynthesis[3] is a potential fermentable carbon source for microorganisms as well as a starting material for synthesis of other carbohydrates.* The biosynthesis of uniformly labeled D-glucose can be accomplished by growing the potato, Solanum tuberosum, var. Sebago, on ^{13}C enriched carbon dioxide as the sole carbon source. No reference was found in the literature in which potatoes formed tubers within a closed system as reported in this work. The tubers of this plant will store D-glucose in polymeric form as starch granules which are easily isolated and can be acid hydrolyzed to the desired monomeric form.

Photosynthetic carbon dioxide fixation into carbohydrate is accomplished by the ribulose 1, 5-diphosphate carboxylase reaction. This is part of a cyclic pathway involving transketolations and transoldolations in which a carbon atom from fixed CO_2 can be incorporated into the C-3 or C-4 atoms of fructose 6-phosphate during the first cycle. One-fifth of the fructose 6-phosphate is available for diversion into starch, while the remainder is recycled back into ribulose 1, 5-diphosphate containing initially fixed CO_2 in the C-1, C-2, and C-3 carbon atoms. Thus, fixed CO_2 will appear in all carbon atoms of fructose 6-phosphate and therefore all carbon positions of starch as early as the second cycle. Once the photosynthetic CO_2 fixation system is in an equilibrium environment of an isotopic CO_2 mixture, a rapid equilibrium of isotopic carbon incorporation into starch can be anticipated.

EXPERIMENTAL

The growth chamber was assembled from two stainless steel glove boxes placed end to end. The overall chamber was approximately two meters long, one meter high and averaged one

*Dr. B. M. Tolbert of the University of Colorado is using the ^{13}C labeled starch granules as starting material for the synthesis of ascorbic acid.

half meter wide; it was fitted with two 90 cm by 90 cm by 6 mm lucite front windows and two 90 cm by 30 cm by 6 mm lucite top windows. The plants were illuminated from the exterior with twelve Sylvania cool white, very high output, fluorescent lamps 193 cm in length. Additional illumination was provided by eight 40 W incandescent bulbs located above the top windows. The light intensity at the potato plant measured about 5,000 fc. A photograph of the chamber with the front lights raised is shown in Fig. 1.

The temperature within the chamber was controlled by a refrigeration unit made from an automobile air conditioner. During each 24 hour period the lights were on for 12 hours with the temperature at 21°C and off for 12 hours with the temperature at 13°C.

The carbon dioxide was supplied to the growth chamber from a 250 cm^3 cylinder containing 150 g of carbon dioxide labeled to 31 at.% ^{13}C (a factor of 28 above the normal 1.1 at.% ^{13}C). The cylinder was refilled every 10 to 20 days.

The CO_2 level was controlled at 850 ppm $\pm$ 30 ppm. A portion of the atmosphere from the chamber was circulated through a Beckman 315A Infrared Analyzer. The signal from the analyzer was transmitted to a recorder-controller which in turn opened a solenoid valve when the carbon dioxide level dropped below the desired concentration, thus returning the carbon dioxide level to 850 ppm.

A 15 cm high potato plant which had been growing in a greenhouse for 31 days was chosen as the starting material for the ^{13}C labeling experiment. The plant was contained in an 8 liter plastic vessel** filled with 60 mesh vermiculite. Once each day 4 liters of complete nutrient solution[4] were pumped into the container. This solution drained to a reservoir below the plastic vessel which contained 20 liters of nutrient solution. The nutrient solution was changed weekly by means of external connectors.

**In later experiments metal containers have been used to prevent rupturing of the container during tuberization.

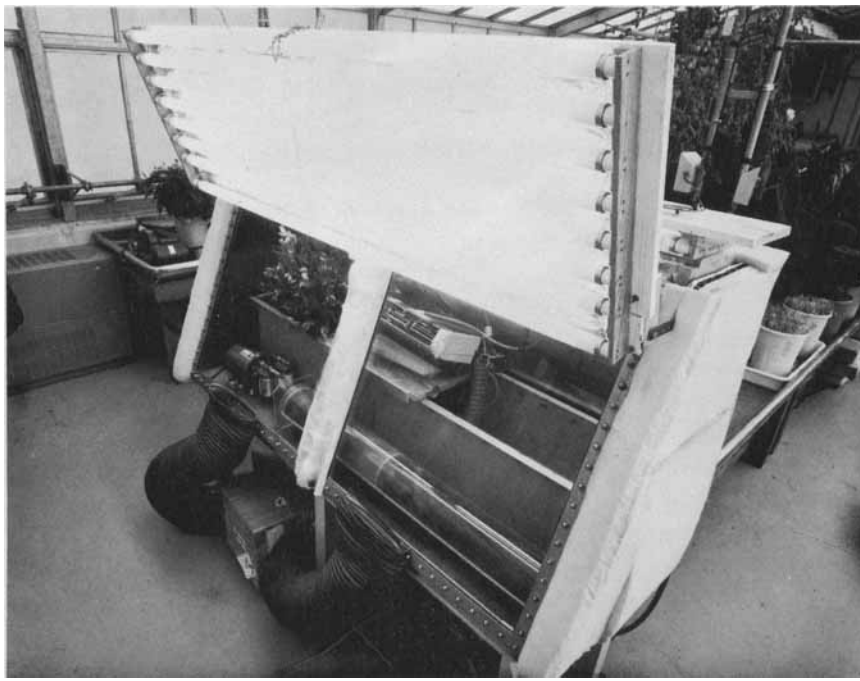

Fig. 1 Photograph of Growth Chamber

A purification column 15 cm in diameter and 120 cm long with one 60 cm section of activated carbon and another 60 cm section of $KMnO_4$ on Al_2O_3 *** was placed inside the chamber. A pump also located within the chamber circulated the atmosphere through the column at 30 liters per minute.

Growth and tuberization of the potato plant appeared normal. After 14 days in the growth chamber the vine had grown to 45 cm. The leaves and blossoms were typical of a healthy potato plant.

One tuber with a wet weight of 126 g was harvested 43 days after the ^{13}C carbon dioxide addition had begun. A cen-

***Trade name - Purafil, obtained from H. F. Burroughs and Assoc., Inc., P. O. Box 80434, Chamblee, Georgia 30341.

tral core was sectioned into eight one centimeter pieces which were analyzed for ^{13}C content. The results are shown in Fig. 2. The remaining tubers were harvested 80 days after the ^{13}C carbon dioxide addition had begun. Anlaysis of 14 small sections from one of these tubers varied randomly from 28.2 to 30.2 at.% ^{13}C and averaged 29.2 at.% ^{13}C. The total yield of tubers was 1,684 g fresh weight. The vines and roots had a fresh weight of 1,340 g. Distribution of fixed carbon based on elemental analysis of lyophylized samples was as follows: 67% in the tubers, 29% in the vines and roots and 4% lost in the nutrient solution.

The growth chamber atmosphere and the potato plant leaves were sampled each week after the addition of the ^{13}C labeled carbon dioxide had begun. These results are given in Figs. 3 and 4.

Starch was isolated by the method of Schoch.[5] Potato tubers weighing 1,451 g were sliced into small pieces and homogenized in a variable speed Waring Blender with 2 ml water per gram of fresh weight. Initial homogenization was for five minutes using a low rotor speed, followed by filtration through four layers of cheesecloth. Extraction of the residue with water using this procedure was repeated four times, increasing the rotor speed with each homogenization.

Filtration of the final homogenate left a residue of fibrous, water-insoluble material. The filtrates from the extractions were pooled and the starch granules allowed to settle out. Most of the granules settled within an hour but the recovery was increased 15% by allowing the filtrate to stand overnight. This procedure separated the filtrate into a starch granule fraction and a water-soluble fraction. The water-soluble fraction, which contains the monomeric and low molecular weight polymers of D-glucose, was decanted from the starch granule sediment, concentrated under vacuum at 40°C and freeze-dried. This fraction weighed 32 g and from enzymatic analysis contained 14 g of glucose, averaging 29.3 at.% ^{13}C.

The starch granule fraction was resuspended in water and filtered through four layers of cheesecloth. After the granules in the filtrate had settled, the washing procedure

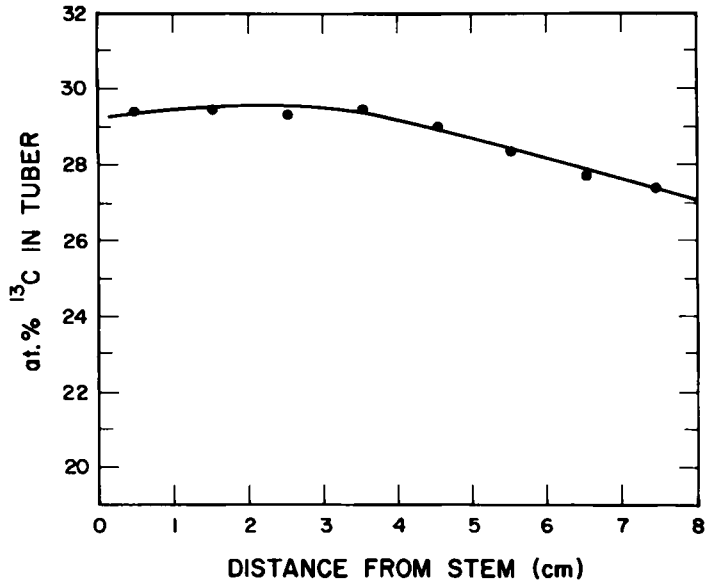

Fig. 2. Distribution of ^{13}C in tuber harvested after 43 days.

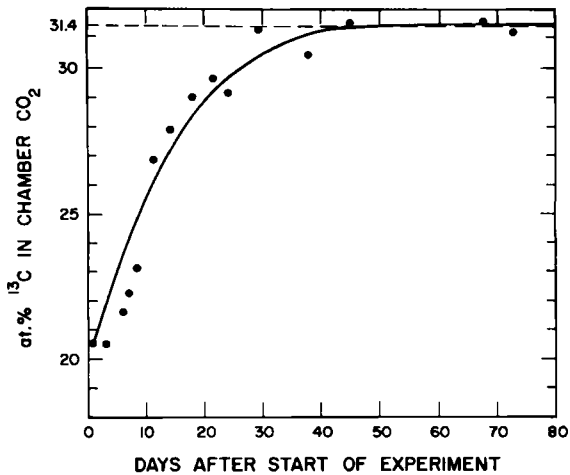

Fig. 3. Variation of ^{13}C in chamber with time.

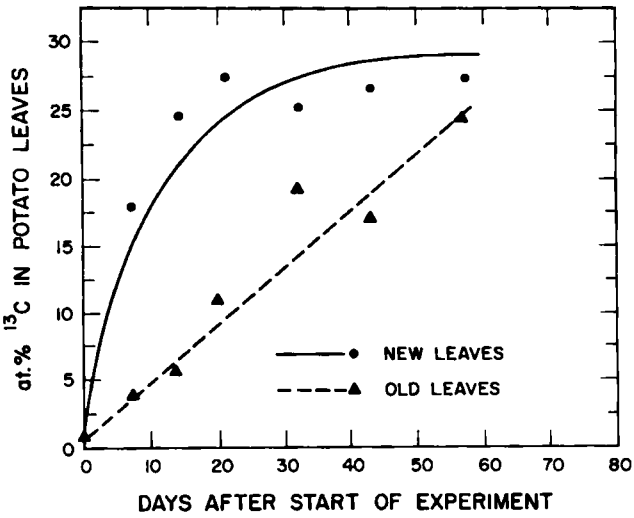

Fig. 4. Variation of ^{13}C in potato leaves with time.

was repeated with water followed by methanol. The washed granules were suspended in methanol, collected on Whatman #2 filter paper, and dried by vacuum for 10 minutes. The granules weighed 145 g and from enzymatic analysis contained 88 g of glucose averaging 28.6 at.% ^{13}C.

A total of 1,087 g of ^{13}C labeled CO_2 was used in the experiment including an estimated 260 g lost due to a leak in the pressure reducing system. Taking into account that starch granules were isolated from only 1,451 g of the tubers harvested, this represents a 16% yield of glucose from the granules and the water-soluble fraction.

Carbon isotope compositions were obtained by wet ashing samples to CO_2 for mass spectroscopy. Proton decoupled ^{13}C nuclear magnetic resonance spectra were obtained at 25.2 mHz on a Varian XL-100 spectrometer interfaced to a Data General Supernova computer using the Fourier transform mode. Free induction decays of 40 μsec rf pulses were accumulated as 8,192 data points in the time domain and transformed into 4,096 points in the frequency domain. The field was stabilized

using the deuterium resonance of D_2O as a lock. Chemical shifts have been determined relative to external methanol.

A 0.2 g sample of starch granules was boiled in 10 ml of D_2O and transferred to an NMR tube. After cooling, the resultant gel was used directly for NMR analysis. The NMR spectrum of the starch granule fraction is given in Fig. 5, A.

A 2.0 g sample of the dried starch granule fraction was suspended in 8 ml of 0.1 N H_2SO_4 and autoclaved under pressure at 120°C for 60 minutes. The hydrolyzed solution was neutralized with $Ba(OH)_2$ to precipitate the sulfate anion as $BaSO_4$, leaving an aqueous solution of D-glucose. This solution was diluted with an equal volume of D_2O for nuclear magnetic resonance analysis. This spectrum is illustrated in Fig. 5, B.

DISCUSSION

A 31 day old plant was used as a starting point for the experiment in order to minimize the time necessary to obtain tubers while still achieving the degree of labeling necessary for subsequently planned NMR and tracer studies. Although an effort was made to purge the box and purification column of normal isotopic abundance CO_2, the ^{13}C level in the box did not attain that of the carbon dioxide supply until after 30 days (Fig. 3).

^{13}C analysis of the first tuber, harvested after 43 days of ^{13}C carbon dioxide addition, indicated a lower ^{13}C content at the end opposite the stem (Fig. 2). It is possible that starch in this region of the tuber was formed before the atmosphere of the box had reached the maximum 30 - 31 at.% ^{13}C level. After an additional 37 days, uniformity of ^{13}C content in the tubers harvested could be supporting evidence for the mobility of carbohydrates within a potato tuber.[6,7] However, even those tubers harvested at the end of the experiment (29.2 at.%) did not reach the ^{13}C content of the carbon dioxide supply (31 at.%). This could be due in part to the ^{12}C present when the experiment started (Fig. 3).

As shown in Fig. 4, the potato vine did not reach equilibrium

with the carbon dioxide supply, and this might be expected to contribute to the lower ^{13}C in the tubers.

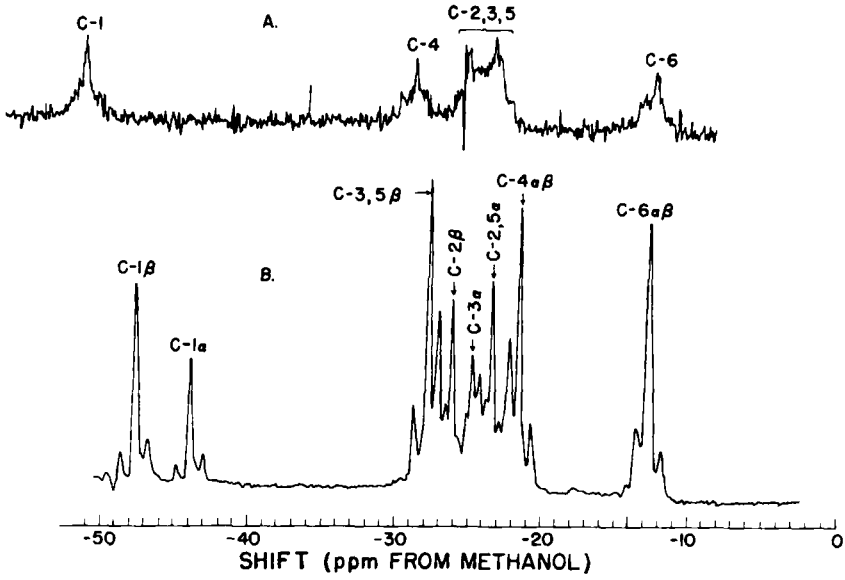

Fig. 5. ^{13}C NMR spectra of starch granule derivatives

(A) Starch gel in D_2O, from 1,000 pulses

(B) Hydrolyzed starch granules in 50% D_2O, from 500 pulses

The first potato plants grown in the sealed chamber with normal isotopic carbon dioxide grew poorly and failed to produce tubers. Since growth returned to normal when the chamber was ventilated with outside air, accumulation of some toxic substance was suspected. Ethylene was detected at 58 ppb in the atmosphere of a sealed chamber, but proof that it is the toxic agent awaits further experiments. It is possible that molds found growing on the surface of the vermiculite were the source of ethylene. No problems with growth or tuberization have been encountered since installation of the purification column.

In previous studies with algae and yeast,[8, 9, 10, 11] no adverse changes were observed with labeling up to 93 at.% ^{13}C. The potato plant supplied with 31 at.% ^{13}C labeled CO_2 grew and tuberized at a rate indistinguishable from those grown on normal CO_2. However, in the present work no simultaneous control was provided, hence small differences in growth rate might not have been detected. The appearance of the tubers and plants was also indistinguishable from those containing normal ^{13}C.

The ^{13}C NMR spectrum of the starch granule fraction (Fig. 5, A), corresponds to that reported for soluble starch by Dorman and Roberts[12] and peak assignments are made using their arguments. Isotope distribution is difficult to ascertain from this spectrum because of the limited concentration and the peak broadening due to the unfavorable molecular correlation time for the gel phase. However, hydrolysis of the polymeric starch granules to monomeric glucose units overcomes both of these problems. Hydrolyzed starch granules exhibit a ^{13}C NMR spectrum (Fig. 5, B) typical of D-glucose, consisting of an equilibrium mixture of the α and β anomers. Peak assignments based on the arguments of Dorman and Roberts[12, 13] and the relative integrals of these peaks are listed in Table I. The latter spectrum shows that the equilibrium mixture contains 65.5% β anomer and 34.5% α anomer. The respective values for normal isotopic glucose would be 63.8% β and 36.2% α as determined by rotation or 62.6% β and 37.4% α as determined by oxidation.[14] Due to experimental uncertainty the values for α and β anomers of ^{13}C enriched glucose as determined by NMR are not significantly different from the values of normal glucose given by other methods. The internal distribution of ^{13}C in terms of relative peak integrals is 17% ± 1% of the total in C-1, 16% ± 1% in C-4, 16% ± 1% in C-6 and an average of 17 ± 1% for C-2, C-3, and C-5 which peaks were not resolved. An accurate estimate of the individual carbons of this last group is difficult because of the carbon-carbon spin splitting and the relatively small differences in chemical shift among their resonances.

TABLE I

Carbon Species	Peak Positions ppm Upfield from Methanol		Peak Integrals
	-11.7		
C-6αβ	-12.6	J_{C-C} = 41.5 Hz	32,048
	-13.3		
	-20.6		
C-4αβ	-21.5		31,744
	-22.1		
	-22.8		
C-2, 5α	-23.3		
	-23.7		
	-24.2		
C-3a	-24.6		
	-25.4		101,388
	-25.6		
C-2β	-26.0		
	-26.5		
	-26.9		
C-3, 5β	-27.7		
	-28.6		
	-43.0		
C-1α	-43.9	J_{C-C} = 44.9 Hz	11,954
	-44.8		
	-46.8		
C-1β	-47.7	J_{C-C} = 46.7 Hz	22,669
	-48.6		

Table I. Relative Abundance of ^{13}C Within Glucose Molecules

Peak assignments for noncoupled carbons are based on arguments of Dorman and Roberts[6, 7]. For carbons with only one carbon neighbor, the coupling constants (J_{C-C}) are given. Peak integrals are from digital integration by computer.

Uniform isotopic composition at each carbon position is important if starch is to be used as a carbon source for the biosynthesis of randomly enriched microorganisms or biomolecules. Since very little carbon metabolism goes through a one carbon intermediate, random enrichment requires a randomly enriched starting material. The NMR spectrum of the glucose obtained by starch hydrolysis (Fig. 5, B) shows the ^{13}C content of the individual carbon positions to be similar on the basis of relative peak integrals. Factors such as minor amounts of compounds other than D-glucose in the starch hydrolysate and potential differential nuclear Overhauser enhancements from proton decoupling prevent a direct correlation of relative peak integral with relative ^{13}C content. However, the peak integral data presented in Table I do not indicate any substantial nonuniformity of ^{13}C enrichment among the various carbon positions.

The 102 g of glucose from the 1,451 g of fresh tubers is less than might be expected. Since the residue remaining after the starch extraction was to be utilized for the growth of microorganisms, the method of starch isolation was chosen for its simplicity. Complete recovery of the starch was not attained.[5] This, along with the loss of CO_2, was responsible for the relatively poor glucose yield (16%). Using average values for starch found in potato tubers[5], yields of 40-50% glucose should be attainable.

The method of biosynthesis of labeled compounds reported here has several advantages over other methods. By starting with seeds or seed pieces it should be possible to attain a higher degree of labeling than by photosynthesis using excised leaves.[3] A uniformly labeled substrate for the growth of microorganisms could be provided by a direct hydrolysis of the tubers, thus saving an isolation step. An almost endless variety of labeled compounds could be prepared by this method since almost any plant could be grown to the desired level of labeling. Finally a growth chamber lends itself easily to the economics of scale up; 10 to 100 times the quantities of labeled starch obtained in this work could be produced with the existing technology.

The growth of potatoes in a closed system can also be used for the biosynthesis of substrates low in ^{13}C. Organisms grown on these substrates will also be depleted in ^{13}C, hence NMR methods could be used to follow the incorporation into the organism of readily available compounds which contain normal isotopic ^{13}C.

Two potato plants have been grown and have tuberized successfully in the apparatus described above using a CO_2 supply containing .0039 at.% ^{13}C, (a factor of 280 below the normal 1.1 at.% ^{13}C). This experiment is continuing; starch has not been isolated and isotopic analysis has not been completed.

ACKNOWLEDGMENTS

The authors wish to thank D. J. Martinez and E. Martinez, Jr. for their technical assistance, T. R. Mills for the mass spectrometer analysis, N. A. Matwiyoff for the use of the NMR, V. H. Kollman for the enzymatic glucose analysis, and B. M. Tolbert of the University of Colorado for his continuing interest and encouragement.

REFERENCES

1. Armstrong, D. E., Briesmeister, A. C., McInteer, B. B., and Potter, R. M., Los Alamos Scientific Laboratory report LA-4391, April 1970.

2. Hammond, A. L., Science, 176, 1972, pp. 1315-1317.

3. Abraham, S. and Hazzid, W. Z., Methods in Enzymology, Vol. IV (Colowick, S.P. and Kaplan, N. O., Eds.), Academic Press, Inc., N. Y., 1957, p. 489.

4. Houghland, G. V. C., American Potato Journal, 24, 1952, pp. 257-62.

5. Schoch, T. J., Methods in Enzymology, Vol. III, (Colowick, S. P. and Kaplan, N. O., Eds.), Academic Press, Inc., N. Y., 1957, p. 5.

6. Moorby, J., Ann. Bot. (London), 32, 1968, pp. 57-58.

7. Moorby, J., Ann. Bot. (London), 34, 1970, pp. 297-308.

8. Fowler, E. B. and Kollman, V. H., Los Alamos Scientific Laboratory report, LA-4496, May 1970.

9. Kollman, V. H., Adams, W. H., Buchholz, J. R., Christenson, C. W., Langham, J., Price, R., and Fowler, E. B., Los Alamos Scientific Laboratory report LA-2551, September 1970.

10. Buchholz, J. R., Adams, W. H., Christenson, C. W., Kollman, V. H., Johnson, G. L., and Fowler, E. B., Los Alamos Scientific Laboratory report LA-4902, April 1972.

11. Fowler, E. B., Adams, W. H., Christenson, C. W., Kollman, V. H., and Buchholz, J. R., Biotechnol. Bioeng., 14, pp. 819-829, 1972.

12. Dorman, D. E., and Roberts, J. D., J. Amer. Chem. Soc., 93, 1971, pp. 4463-4472.

13. Dorman, D. E., and Roberts, J. D., J. Amer. Chem. Soc., 92, 1970, pp. 1355-1361.

14. Pigman, W., The Carbohydrates, Chemistry, Biochemistry and Physiology, Academic Press, Inc., N. Y., 1957, p. 52.