

**Purchased by
Agricultural Research Service
U. S. Department of Agriculture
For Official Use**

2089

THE GLUCOSE OXIDASE OF HONEY

I. PURIFICATION AND SOME GENERAL PROPERTIES OF THE ENZYME

ABNER I. SCHEPARTZ AND MARY H. SUBERS

Eastern Regional Research Laboratory, Philadelphia, Pa. (U.S.A.)*

(Received October 28th, 1963)

SUMMARY

A glucose oxidase has been isolated from honey and partially purified. A study of its general properties indicates a strong similarity to the enzyme occurring in the honeybee. It is characterized by a high substrate specificity and an unusually high substrate-concentration requirement.

INTRODUCTION

There are many enzymes capable of catalyzing the oxidation of glucose to gluconic acid. Most of these act by transferring hydrogen anaerobically to either NAD or NADP and are called "glucose dehydrogenases" (β -D-glucose: NAD(P) oxidoreductase, EC 1.1.1.47). Those few enzymes that do not require NAD or NADP, but instead function aerobically, transferring hydrogen directly to molecular O_2 , are known as "glucose oxidases" (β -D-glucose: O_2 oxidoreductase, EC 1.1.3.4). With one exception, the latter type have been found only in plant tissue, never in animal tissue. Thus glucose oxidases have been reported present in molds¹, a red alga², citrus fruits³, and bacteria⁴. The one exception is the pharyngeal gland of the honeybee⁵.

In a recent study on the antibacterial factor in honey known as "inhibine"⁶, we have shown that this factor was H_2O_2 , being produced by the action of a glucose oxidase in the honey. This finding strongly paralleled the earlier work on the mold enzyme^{7,8} and its antibiotic property, originally called "notatin" or "penicillin-A", which was also due to H_2O_2 . The present report is based on some studies of the fundamental nature of the glucose oxidase in honey.

MATERIALS AND METHODS

The enzyme source was an unheated, Northeastern fall-flower honey (No. HS-37) that had been stored at 4°.

Sephadex G-100** was obtained from Pharmacia Fine Chemicals, peroxidase

* Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

** Mention of trade or company names does not imply endorsement by the Department over others of a similar nature not named.

(donor: H_2O_2 oxidoreductase, EC 1.1.1.7) from Sigma Chemical Company, and *o*-dianisidine from Eastman Organic Chemicals.

Brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) was prepared by the method of TISELIUS⁹.

Standard Warburg manometric procedures were employed in experiments where O_2 uptake was determined. The main space of the vessel contained substrate in buffer. The enzyme was introduced from a side arm sac. Alkali was present in the center well.

Enzymatic activity was also determined by the production of H_2O_2 and its subsequent reaction with peroxidase and *o*-dianisidine⁶. The standard test system consisted of the following, in a total of 3.5 ml: 1.5 ml glucose (3.5 M dissolved in 0.2 M sodium phosphate (pH 6.1)); 1.8 ml phosphate buffer (0.2 M sodium phosphate (pH 6.1)); 0.1 ml *o*-dianisidine (3.5 mg/ml in 95% ethanol); 1 drop peroxidase (0.04 mg/ml in 0.2 M sodium phosphate (pH 6.1)); 0.1 ml enzyme solution of unknown activity. Incubation was for 15 min at 37°. The reaction was then halted sharply by addition of 1 drop of conc. HCl, dropping the pH to 2. After standing for an additional 15 min at room temperature, the absorbancy was read at 402 $m\mu$ on a Bausch and Lomb, model 505, spectrophotometer. Calibrated against known H_2O_2 , this absorbancy was linear over the range from 0.1 $\mu\text{g/ml}$ to 3.0 $\mu\text{g/ml}$. At least two blanks were always run, one without enzyme and one without glucose. In certain experiments, where glucose was desired in the blanks, peroxidase was withheld. Ordinarily the complete system was incubated together, since none of the reagents was toxic or inhibitory toward the enzyme. The high concentration of glucose necessitated the use of a vortex-type agitator for rapid stirring of assay tubes.

A qualitative/semi-quantitative modification of the above spectral method was useful as a screening technique where many fractions or compounds had to be tested.

This was performed in a spot-plate with incubation by partial submergence in a water bath. The components of this system were (total volume 6 drops): 1 drop enzyme solution or fraction; 2 drops phosphate dye (0.05 mg *o*-dianisidine/ml in 0.2 M sodium phosphate (pH 6.1)); 2 drops glucose (3.5 M in 0.2 M sodium phosphate (pH 6.1)); 1 drop peroxidase (0.04 mg/ml in 0.2 M sodium phosphate (pH 6.1)). Incubation was for 15 min at 37° after which results were judged visually on the basis of color production compared with suitable controls.

Protein was generally determined by the spectral method of WARBURG AND CHRISTIAN¹⁰ as modified by LAYNE¹¹. Certain samples were also run by the method of LOWRY *et al.*¹². Results of the two methods showed good correlation.

One unit of glucose oxidase was defined as that amount of enzyme catalyzing the production of 10^{-3} μmole H_2O_2 per min or the utilization of 10^{-3} μmole O_2 per min at 37°, optimum pH (6.1), and optimum substrate concentration (1.5 M). Specific activity was expressed as enzyme units per mg protein.

EXPERIMENTAL AND RESULTS

*Purification of the enzyme**

100 g of honey were dissolved in 48 ml H_2O , sterilized by incubation with 24 mg Merthiolate for 30 min, 37°, in dialysis tubing, and then dialyzed against running tap

* Small samples were removed for analysis at various points in the procedure.

water (approx. 22°) for 24 h. These operations were performed in the dark or, at most, in subdued light to avoid possible inactivation¹³. Upon removal from the sac, the dialyzed solution was diluted to 200 ml, cooled to 4° (all subsequent operations were carried out at 4° unless otherwise stated), and treated with the slow addition of cold acetone, with stirring, over a period of 30 min, to give 50% acetone by volume. A heavy precipitate formed and was stirred an additional 15 min to assure complete reaction. The suspension was centrifuged at 1500 rev./min (414 × *g*) for 10 min; most of the supernatant was poured off and the residues combined and respun for 10 min. The supernatant was discarded and the precipitate resuspended and washed out of the centrifuge bottle with a total of 5 ml of 0.2 M sodium phosphate (pH 6.1), and into a dialysis sac. This dark brown suspension (20 ml) was dialyzed 2 h against distilled water, with stirring, then against running tap water for 22 h (at tap-water temperature). The suspension was removed from the sac and centrifuged at 1700 rev./min (530 × *g*) for 1 h. A small precipitate was discarded and the supernatant re-centrifuged at 20 000 rev./min (26 000 × *g*) for 1 h in a Spinco, model L, ultracentrifuge. The supernatant was removed by syringe from between a large pellet and a surface emulsion (apparently containing beeswax). The emulsion was respun under the same conditions for 30 min. The supernatant from this was recombined with the major one, total volume 20.1 ml. The pellets and remaining emulsion were discarded.

A Sephadex G-100 column was prepared by allowing 5 g of the gel to swell in water for 48 h. The fines were removed by repeated washings during this period. The gel was poured into a column to give a bed volume of 83 ml (2.2 cm × 22.1 cm) with a holdup volume of 25 ml. After additional water-washing (200 ml), 19.6 ml of the above supernatant were placed on the column. Development with water produced 4 bands which were also eluted with water. The second band gave a dark-brown eluate (17.3 ml), containing the bulk of the oxidase activity. This step was performed at room temperature.

The above Sephadex fraction (16 ml) was transferred to a brushite column (1.2 cm × 15.5 cm, holdup volume 18.5 ml). After development and elution of considerable protein (as well as some enzyme activity) with 0.025 M sodium phosphate (pH 6.8), the major portion of the activity was eluted with 0.2 M of the same buffer. This dark-brown fraction (10 ml) represented the most highly purified enzyme thus far obtainable.

TABLE I
PURIFICATION OF GLUCOSE OXIDASE FROM HONEY

Stage of purification	Enzyme activity (units)	Protein (mg)	Specific activity (units per mg protein)	Purification factor	Yield* (%)
1. Original honey	22 400**	5100***	4.4	1.0	100
2. Dialyzed solution	22 400	1230	18.2	4.1	100
3. Acetone precipitate	18 500	967	19.1	4.3	83
4. 26 000 × <i>g</i> supernatant	17 500	692	25.3	5.8	81
5. Sephadex G-100 fraction	10 300	330	31.2	7.1	49
6. Brushite fraction	5 260	100	52.6	12.0	25

* Corrected for removal of samples for analysis.

** Activity of original honey assumed same as dialyzed solution (see DISCUSSION).

*** Apparent protein (see DISCUSSION).

This purification was devised from experimentation on pilot lots at each stage. The final procedure was then applied to 2 complete batches which gave essentially the same results. The quantities mentioned above are from one of these. Table I lists the analytical data obtained during the process.

Products of the reaction

The enzyme catalyzes the oxidation of glucose to H_2O_2 and gluconic acid (or its actone). This was established in our previous publication⁶.

Effect of pH and type of buffer

The change in activity with variation in pH was determined with the following buffers: sodium phosphate, potassium phosphate, sodium citrate, and Tris-malate, using a partially purified fraction as enzyme source in the peroxidase-dye procedure. All buffers were used at the same ionic strength ($I = 0.6$). The results are shown in Fig. 1.

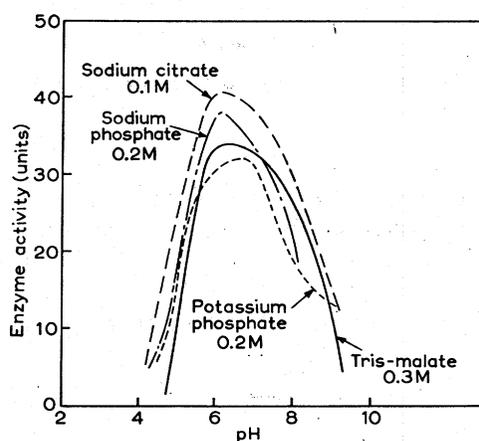


Fig. 1. Effect of pH on enzyme activity using various buffers ($I = 0.6$).

Except for potassium phosphate, the pH optima were all at 6.1. Sodium phosphate was selected as the buffer of choice. Although citrate appeared to afford greater activity, it was discovered that it caused an auto-oxidation of the glucose.

Requirement for Na^+

During the study on buffers it was noted that the enzyme required a buffer containing Na^+ for maximal activity and that the minimum concentration of Na^+ necessary was 0.1 M. Henceforth all test systems were designed to contain approx. 0.2 M sodium phosphate, providing a Na^+ concentration well over the minimum.

Enzyme concentration

The relation between activity and enzyme concentration was linear over a considerable range, as indicated in Fig. 2.

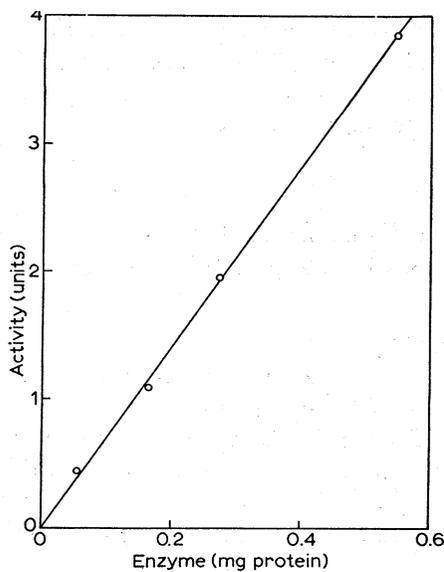


Fig. 2. Effect of enzyme concentration on activity.

Substrate concentration

The effect of substrate concentration on velocity of the reaction was very unusual. Unlike most of the known glucose oxidases the honey enzyme required an extremely high concentration of glucose, the optimum being 1.5 M. This compares favorably with the bee enzyme, which requires 2 M (ref. 5). The velocity-substrate curve, as determined manometrically, is shown in Fig. 3. Similar data were obtained with the

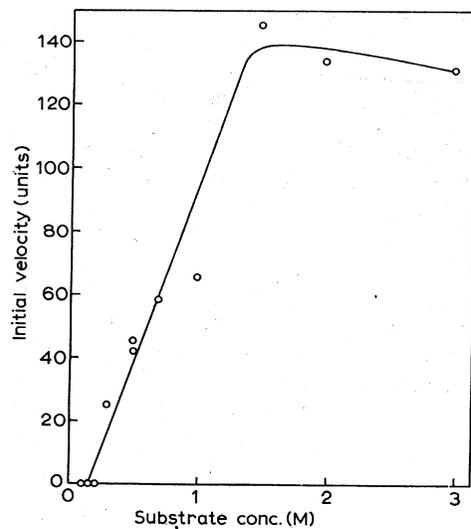


Fig. 3. Effect of substrate concentration on velocity.

peroxidase-dye method. Further details in regard to the reaction kinetics will be reported later.

Substrate specificity

Since the glucose requirement of the enzyme was unusually high, the possibility of a more appropriate substrate was investigated. A series of compounds was checked for activity by the spot-test technique. Any showing promise, in addition to some of the more common sugars in honey, were then tested quantitatively by the peroxidase-dye method. The substrate concentrations employed in both methods were in terms of glucose equivalents or apparent equivalents. Of all the compounds tried, only mannose exhibited significant oxidation and this was 9% of the rate shown by glucose. The results are given in Table II.

TABLE II
SUBSTRATE SPECIFICITY

<i>Oxidized:</i>		
D-Glucose	100	(100 = Standard)
D-Mannose*	9	
<i>Not oxidized:</i>		
<i>Sugars</i>	<i>Sugar derivatives</i>	<i>Sugar polymers</i>
D-Arabinose	L-Ascorbic acid	Amylose
L-Arabinose	Gluconic acid	Amylopectin
Cellobiose	δ -Gluconolactone	Dextrin
D-Fructose	D-Glucosamine	Ethyl cellulose
Galactose	Glucose 1-phosphate	Starch
Lactose	Glucose 6-phosphate	
Maltose	Glucuronic acid	
Melezitose	2-Ketogluconate	
Melibiose	5-Ketogluconate	
Raffinose	α -Methyl glucoside	
Sucrose	D-Sorbitol	
D-Trehalose		
Turanose		
D-Xylose		

* The mannose contained no detectable glucose when tested by paper chromatography in a system sensitive to the presence of 0.3% glucose.

Since H_2O_2 can also be produced by certain amino acid oxidations and since proline¹⁴ and pyroglutamic acid¹⁵ have been reported in honey, several of these acids were also tested for substrate activity. These were glycine, L-glutamic acid, D-aspartic acid, L-proline and L-pyroglutamic acid. No significant activity was found.

Effect of temperature

The relationship between enzyme activity and incubation temperature was studied using the peroxidase-dye method at a variety of temperatures from 25.5° to

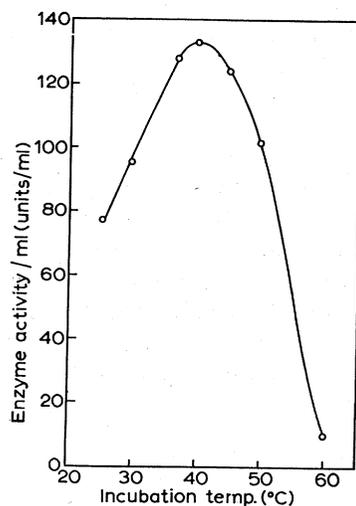


Fig. 4. Effect of incubation temperature on enzyme activity.

60°. As shown in Fig. 4, 40° was optimal. Use of proper controls indicated that the peroxidase test-system remained uninjured up to 60° during these experiments.

The temperature stability of the enzyme itself was investigated by heating a solution of enzyme for 5 min at a specific temperature, cooling rapidly, then assaying by the peroxidase-dye reaction. At 60° the enzyme was completely inactivated. As seen in Fig. 5, 50° appeared to be critical in that maximum changes occurred in that area. An inactivation-time curve at 50° is given in Fig. 6.

Inhibitors and activators

A number of compounds were tested as possible enzyme inhibitors. In addition to some of the common honey sugars, these were materials of interest reported

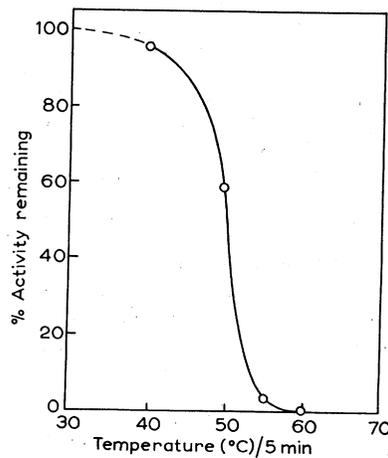


Fig. 5. Effect of temperature on enzyme inactivation.

HONEY GLUCOSE OXIDASE. I. PURIFICATION AND PROPERTIES

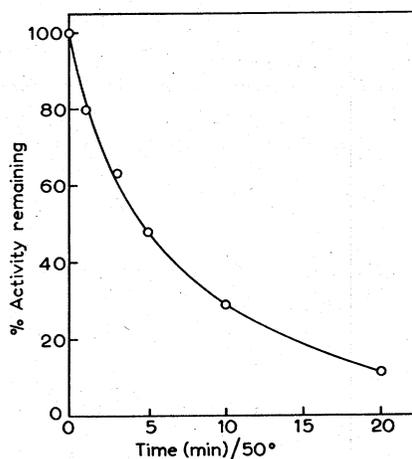


Fig. 6. Effect of time on enzyme inactivation at 50°.

previously in inhibition studies of other glucose oxidases^{2,3,5,7}. The sugars were tested at 0.09 or 0.1 M to be comparable with mannose, which had been reported as inhibitory by BEAN and coworkers^{2,3}. Gluconate and δ -gluconolactone were run at the concentration expected for reaction products (based on H_2O_2 production). $(NH_4)_2SO_4$ was tried because of its inactivation of the enzyme during purification attempts. All other materials were tested at concentrations reported in the literature. The peroxidase-dye method was used, including appropriate controls to assure no effect on the peroxidase or dye. The results are listed in Table III. Further work along these lines is in progress.

Above the mild requirement for Na^+ , we have failed to find any specific, dramatic

 TABLE III
 ENZYME INHIBITION

<i>Inhibitors</i>	<i>Concn. (M)</i>	<i>% inhibition</i>
NaCN	0.001	94
Semicarbazide	0.01	90
EDTA	0.007	14
Mannose	0.09	12
Fructose	0.1	18
NaN_3	0.002	8
<i>Did not inhibit:</i>		
At 0.1 M: sucrose, maltose, xylose		
At 0.01 M: benzoate, nitrate, gluconate, δ -gluconolactone, gluconolactone mixture (acid, δ , γ)		
At 0.001 M: Ag^+ , Pb^{2+} , Hg^{2+} , Fe^{3+} , Ba^{2+} , Cu^{2+} , Co^{2+} , Mg^{2+} , acetate, propionate, borate, $(NH_4)_2SO_4$		

activation of this enzyme. Mn^{2+} showed a slight effect at 0.004 M and Tris even less at 0.01 M. Addition of FAD was without response.

The influence of O_2 on the reaction, when run manometrically, was easily seen. The activity of the enzyme was more than 2 times as great in pure O_2 as in air, corroborating the findings of KEILIN AND HARTREE on the mold enzyme⁷.

Isoelectric point

Determined by precipitation experiments, the isoelectric point of the enzyme protein was at pH 4.3-4.5. Honey proteins in general are known to be isoelectric at about 4.3 (ref. 16).

DISCUSSION

The glucose oxidase of honey strongly resembles the enzyme found in the honeybee⁵ and may be identical with it. Thus both require extremely high substrate concentration (1.5 M and 2 M, respectively), have pH optima at 6.1 and 6-7, respectively, and act only on glucose (except for a very small mannose activity). Temperature stability and other properties are also similar. The only real point of difference is in inhibition. GAUHE⁵ reported that CN^- was not inhibitory and, in fact, appeared to activate the bee enzyme. In contrast, we find CN^- a strong inhibitor of the honey enzyme.

The other known glucose oxidases generally are quite different in properties from the honey enzyme. Most of them require only 0.1 M concentration of substrate. The mold enzyme⁷, while being rather specific for glucose, has an optimum pH at 5.6, and contains an easily recognizable FAD linkage. Although, like the honey enzyme, it is inhibited by semicarbazide, it remains unaffected by CN^- or azide, but is inhibited by NO_3^- . The enzyme from the red alga² acts on a number of substrates. While it too is inhibited by mannose, it is also inhibited by Hg^{2+} , Pb^{2+} , Ag^+ , benzoate, propionate and acetate, which have no effect on the honey enzyme. The citrus-fruit enzyme³ can catalyze the oxidation of at least nine sugars. It is inhibited by mannose, Cu^{2+} , Ba^{2+} , Hg^{2+} and Ag^+ , and xylose, but is unaffected by azide or acetate. The bacterial enzyme⁴ acts on galactose and glucose esters, as well as glucose, at an optimum pH of 5.5. Thus the latter two enzymes also differ from the honey oxidase.

It was impossible to purify the honey enzyme by the usual $(NH_4)_2SO_4$ or uranyl acetate methods. The former caused complete inactivation; the latter did not effect a precipitation. The use of DEAE- and CM-cellulose failed. The enzyme could not be removed from the DEAE-cellulose without destructive treatment, while it would not adsorb on the CM-cellulose. Among the various types of Sephadex available, G-100 was most suitable. The repeated emergence of the major activity in the second band eluted from this column indicated a molecular weight somewhat below 100 000. This is in contrast with the reported 152 000 for the mold enzyme¹⁷.

Thus far, we have been unable to establish, by spectral examination, the presence of FAD in our preparations. Nor have we found a requirement for it. This may be due to the fact that we are working with much smaller amounts of enzyme than in the case of the mold oxidase.

In Table I the activity of the original honey was assumed to be the same as that

of the dialyzed solution in the next step of the preparation. This was necessitated by the inability to obtain a reproducible value for enzyme activity before dialysis. Undialyzed honey contains many factors influencing the production and destruction of H_2O_2 , whereas the dialyzed honey does not. The assumption does not appear to be in serious error, since one would not expect great losses at this point and none have been detected.

Table I also indicates the presence of a large amount of protein in the original honey which subsequently is reduced on dialysis. This seems highly unlikely for true protein. We, therefore, consider this figure as representing "apparent protein", since it is still a measure of materials capable of absorbancy at $280 m\mu$ and reactivity in the LOWRY method.

The quantitative peroxidase-dye method of analysis was used most extensively because of its great sensitivity. This system could detect as little as 1 enzyme unit per ml in a given sample, whereas the spot-test required about 3 units/ml and the manometric method about 9 units/ml.

The glucose oxidase of honey is not peculiar to the particular type of honey used in these experiments. It has been detected and found to occur in varying amounts in almost all types tested¹⁸.

ACKNOWLEDGEMENTS

The authors are greatly indebted to Dr. J. W. WHITE, JR., for his continued interest and advice. They also thank Dr. L. WEIL for many helpful discussions.

REFERENCES

- ¹ D. MÜLLER, *Biochem. Z.*, 199 (1928) 136.
- ² R. C. BEAN AND W. Z. HASSID, *J. Biol. Chem.*, 218 (1956) 425.
- ³ R. C. BEAN, G. G. PORTER AND B. M. STEINBERG, *J. Biol. Chem.*, 236 (1961) 1235.
- ⁴ J. H. DOWLING AND H. B. LEVINE, *J. Bacteriol.*, 72 (1956) 555.
- ⁵ A. GAUHE, *Z. Vergleich. Physiol.*, 28 (1941) 211.
- ⁶ J. W. WHITE, JR., M. H. SUBERS AND A. I. SCHEPARTZ, *Biochim. Biophys. Acta*, 73 (1963) 57.
- ⁷ D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 42 (1948) 221.
- ⁸ C. E. COULTHARD, R. MICHAELIS, W. F. SHORT, G. SYKES, G. E. H. SKRIMSHIRE, A. F. B. STANDFAST, J. H. BIRKINSHAW AND H. RAISTRICK, *Biochem. J.*, 39 (1945) 24.
- ⁹ A. TISELIUS, S. HJERTÉN AND Ö. LEVIN, *Arch. Biochem. Biophys.*, 65 (1956) 132.
- ¹⁰ O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1942) 384.
- ¹¹ E. LAYNE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 451.
- ¹² O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
- ¹³ J. W. WHITE, JR. AND M. H. SUBERS, in preparation.
- ¹⁴ A. KOMAMINE, *Suomen Kemistilehti B*, 33 (1960) 185.
- ¹⁵ E. E. STINSON, M. H. SUBERS, J. PETTY AND J. W. WHITE, JR., *Arch. Biochem. Biophys.*, 89 (1960) 6.
- ¹⁶ R. E. LOTHROP AND H. S. PAINE, *Ind. Eng. Chem.*, 23 (1931) 328.
- ¹⁷ R. CECIL AND A. G. OGSTON, *Biochem. J.*, 42 (1948) 229.
- ¹⁸ J. W. WHITE, JR. AND M. H. SUBERS, *J. Apicult. Res.*, 2 (1963) 93.