THE EFFECT OF 6-DEOXY-6-FLUORO-D-GLUCOSE ON YEAST FERMENTATION AND ON HEXOKINASE*

by

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Structural analogues of many biologically important compounds have been synthesized and their biological effects studied. However, the effect of glucose analogues on glucose metabolism has received little attention until recently, when MACDONALD and his co-workers began their studies of such analogues[1]. Of about 50 compounds they have tested, 2-deoxy-D-glucose has been found to be the most potent inhibitor of yeast fermentation and of tumor glycolysis[2].

This paper is concerned principally with the effect of 6-fluoro-6-deoxy-D-glucose*** on the fermentation of glucose and fructose by intact yeast and yeast extracts and on the action of yeast hexokinase. In addition, some data are given on the relation of 6FG to the uptake of glucose by rat diaphragm and on other biological effects of 6FG. Interest in the biochemistry of fluoro-derivatives of glucose was prompted by the results of studies with fluoroacetate and related compounds[3,4], the small change in molecular weight when an –OH is replaced by a –F, and the possible usefulness of such derivatives as specific inhibitors of glycolysis. The 6-fluoro derivative was chosen because its preparation had been described[5,6] and the probability that it would be sufficiently stable under biological conditions for the contemplated experiments. The authors are not aware of any biochemical studies with 6FG other than the report of HELFERICH, GRÜNLER, AND GNÜCHTEL that its glycoside is hydrolyzed by emulsin[7].

MATERIALS AND METHODS

6-Fluoroglucose was prepared by a modification of the method of HELFERICH *et al.*[5,6] as described elsewhere[8]. The preparation is tedious and thus limited somewhat the extent of biological studies with the compound. The C–F bond of 6FG is stable to hot acid, but is readily split under alkaline conditions. For example, at room temperature, in Na_2CO_3 solution at pH 10.5, 6FG was one-half decomposed in 6 days as measured by the inorganic fluoride formed.

Brewer's bottom yeast was obtained as a slurry§, filtered by suction to remove most of the liquid, washed several times with 0.87% NaCl solution by centrifugation, filtered to obtain a solid yeast cake, and stored at 6° C. Studies were made with preparations not older than one week. Fleischmann's baker's yeast cake was used as the source of baker's yeast.

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*** Also called glucose-6-fluorohydrin by HELFERICH[5,6]. In this paper it will be abbreviated to 6FG.

§ Kindly supplied by Hamm Brewery Co., St. Paul, Minn.

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Prior to use the yeast preparations were washed 5 or 6 times with a buffer solution (0.079 *M* potassium acetate and 0.034 *M* phosphate at pH 4.6) by centrifugation and resuspended in the buffer. The yeast suspension (0.5 ml containing 6.7 mg of baker's or 10 mg of brewer's yeast) was placed in the main compartment of a Warburg flask and the substrate or 6FG dissolved in buffer was placed in the side arm. The total volume of the contents was 1.1 ml. Fermentation rate at 30° C under an atmosphere of 95% nitrogen and 5% carbon dioxide was determined by the conventional procedures. Rates of fermentation were taken as the slopes of plots of CO_2 produced against time after the maximum rate of CO_2 production had been established following a short induction period. The CO_2 production was sufficiently linear with time to allow determination of reaction rates even at the relatively low glucose concentrations used for some experiments.

Acetone powder preparations of yeast extracts were prepared from brewer's yeast by a modification[9] of the method of HOCHSTER AND QUASTEL[10]. The powder was suspended in 0.067 *M* phosphate buffer (pH 6.15) and 0.3 ml placed in the side arm of a Warburg flask. The metabolite solution, (0.4 ml containing, at pH 6.2, 1 μM of ATP, 0.8 mg DPN, 40 μM of potassium phosphate, 2 μM of magnesium and 50 μM of CH_3CHO), the substrate, and the 6FG were placed in the main compartment and water was added to make the final volume 1.2 ml. Fermentation rate was measured at 30° C under an atmosphere of 95% nitrogen 5% carbon dioxide.

The hexokinase used was a lyophilized preparation obtained from the Pabst Laboratories, Milwaukee, Wis., corresponding to fraction 3a of BERGER, SLEIN, COLOWICK AND CORI[11]. The procedure used for the hexokinase measurements was a described by BERGER *et al.*[11] except that the volume of enzyme solution was reduced to allow for additions of substrate and 6FG. The hexokinase was dissolved in water immediately before use; the amount used corresponded to 0.32 mg per 1.6 ml final volume in the Warburg flask.

RESULTS

Fermentation studies with intact yeast

Preliminary studies showed that 6FG was not fermented by intact yeast, but did inhibit the fermentation of glucose and fructose. Inhibition of the fermentation of glucose was the same with or without preincubation of the yeast with 6FG. Considerable variation in the sensitivity to 6FG was found with different yeast preparations and with storage of the yeast.

Representative results showing the effect of 6FG on the rate of CO_2 production by bakers yeast from different concentrations of glucose and fructose are shown in Fig. 1. They demonstrate a marked inhibition of the fermentation rate by 6FG at low substrate concentrations. At a 1:1 molar ratio of glucose: 6FG the rate of glucose fermentation was inhibited 15% and that of fructose fermentation 52%. The overcoming of the inhibition

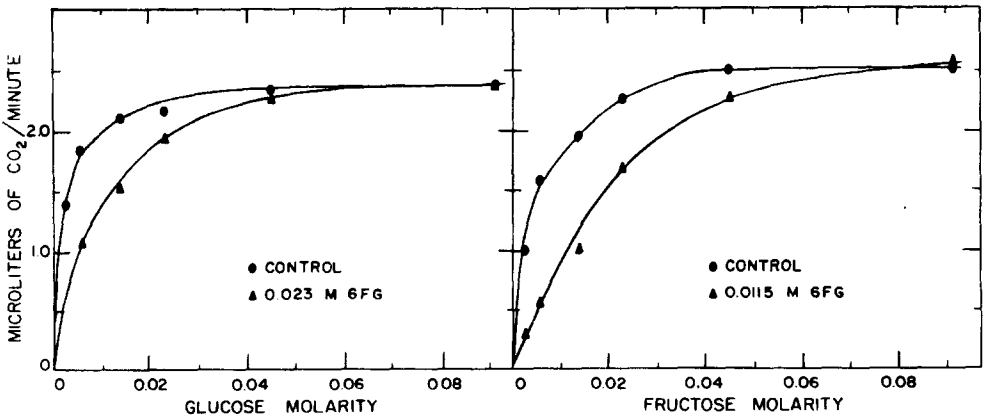

Fig. 1. The effect of increasing concentrations of glucose or fructose on fermentation by intact baker's yeast in the presence of 6-deoxy-6-fluoro-D-glucose (6FG).

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at higher substrate concentrations shows that the inhibition by 6FG may be regarded as competitive. The dependence of the rate of fermentation by the intact yeast on glucose or fructose concentration corresponded fairly closely to that which would be expected from a mass action relation. The relation between fermentation rate and substrate concentration could thus be described by the Michaelis-Menten equation as shown in 1935 by HOPKINS AND ROBERTS[12]. This allowed approximation of values for the "apparent K_m" of glucose and fructose and "apparent K_i" of 6FG using reciprocal plots as suggested by LINEWEAVER AND BURK[13]. From such plots the apparent K_m for glucose was $1.8 \cdot 10^{-3}$ and the K_i $7.3 \cdot 10^{-3}$; for fructose the apparent K_m was $5 \cdot 10^{-3}$ and the K_i $3.3 \cdot 10^{-3}$.

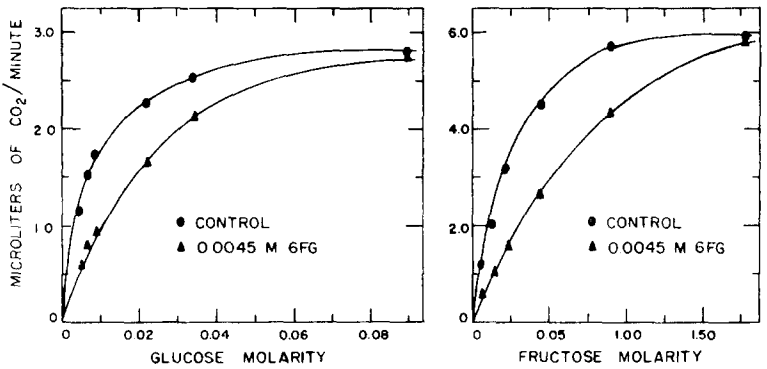

Fig. 2. The effect of increasing concentrations of glucose or fructose on fermentation by intact brewer's yeast in the presence of 6-deoxy-6-fluoro-D-glucose (6FG).

Brewers yeast was somewhat more sensitive to 6FG than baker's yeast. The effect of 6FG on the fermentation of glucose and fructose by brewer's yeast is shown in Fig. 2. As with the baker's yeast, the inhibition was competitive; separate experiments showed conclusively that the inhibition could be overcome by higher levels of substrates than were used for the experiments reported in Fig. 2. From plots of $1/v$ against $1/s$ the apparent K_m value for glucose was $6.9 \cdot 10^{-3}$ and the K_i for 6FG $2.7 \cdot 10^{-3}$; for fructose the apparent K_m was $27 \cdot 10^{-3}$ and K_i $2.3 \cdot 10^{-3}$. At a 1:2 molar ratio of 6FG: substrate the rate of CO_2 production from glucose was inhibited 42% and from fructose 60%.

Fermentation studies with yeast extracts

As with intact yeast, 6FG was not fermented by the yeast extract. In contrast to the results with intact yeast, 6FG did not inhibit the fermentation of glucose or fructose by the yeast extract. The measurable fermentation rate by yeast extract could not be conveniently limited by reduction in substrate concentration; at concentrations sufficiently low to decrease the rate nearly all the substrate was utilized before the initial lag period of the fermentation was complete. The experiments with yeast extract were therefore made with the minimum amount of substrate sufficient for adequate measurement of the fermentation rate. Fig. 3 shows the total CO_2 output obtained from glucose and from fructose with the yeast extract in the presence and absence of 6FG. The data plotted represent the averages of duplicate determinations. In these experiments the molar ratio of 6FG to substrate was 5:1; similar results were also obtained in experiments where the ratio was 6:1. The maximum rate of CO_2 output was nearly the same with or without 6FG present. There was an increase in the lag period in the

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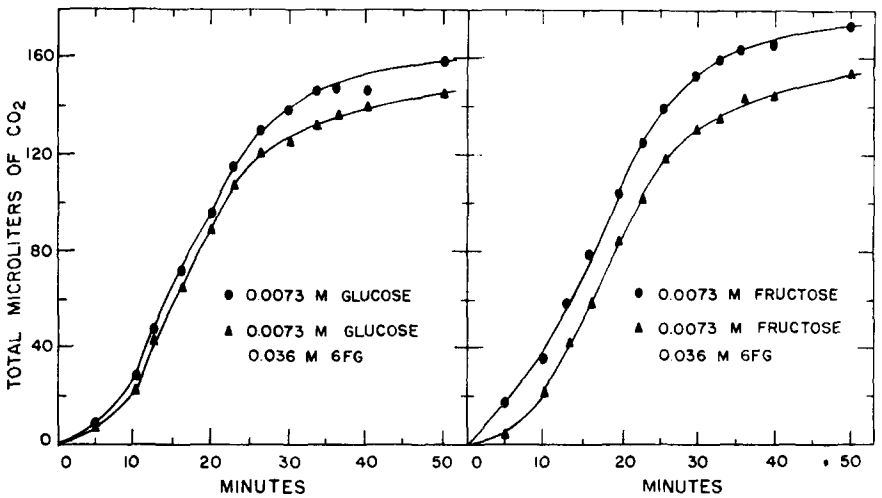

Fig. 3. The effect of 6-deoxy-6-fluoro-D-glucose (6FG) on fermentation of glucose or fructose by a cell-free yeast extract.

presence of 6FG, particularly with fructose as the substrate. This may have resulted from the slight inhibition of hexokinase by the high concentrations of 6FG used. The data show conclusively that 6FG at total concentrations or at ratios of 6FG:glucose which caused a marked inhibition of fermentation by intact yeast had little effect on fermentation by yeast extract.

Hexokinase studies

As would be anticipated from the lack of an –OH group in the 6 position, 6FG was not phosphorylated by hexokinase. Results of experiments to test the effect of 6FG on the action of hexokinase using low levels of glucose and fructose are shown in Fig. 4.

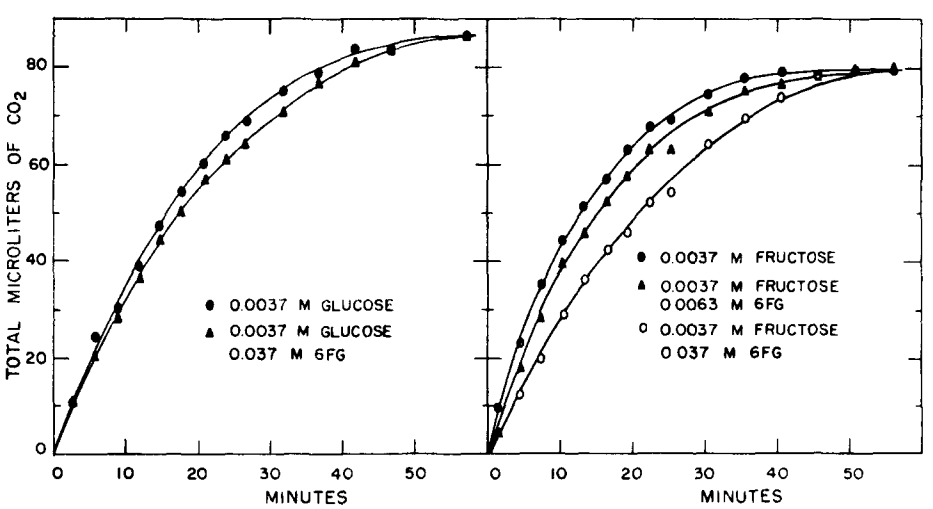

Fig. 4. The effect of 6-deoxy-6-fluoro-D-glucose (6FG) on hexokinase activity with glucose or fructose as substrates.

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When compared on either a total molarity basis or on a basis of the ratio of 6FG: substrate, the inhibition of hexokinase by 6FG was much less marked than that of fermentation by intact yeast. At 0.037 *M* 6FG (ratio of 6FG: substrate equal 10:1) the inhibition of the reaction rate with glucose was less than 10% and that with fructose about 35%; lower 6FG concentrations produced less inhibition. The greater inhibition of fructose phosphorylation is very likely related to the higher K_m of fructose than for glucose in the hexokinase reaction[14].

Miscellaneous biochemical observations with 6FG

Measurements were made on the glucose uptake of rat diaphragm using techniques similar to those of STADIE AND ZAPP[15]. Although considerable variation was encountered in the uptake by the isolated rat diaphragm, a sufficient number of experiments was performed to establish that 6FG had a much smaller inhibitory effect on the uptake of glucose by the diaphragm than on glucose fermentation by intact yeast. The average uptake of 6FG by diaphragm, unlike that of glucose, reached a maximum value within 1 hour; the total uptake after 2 hours (average = 0.53 mg per gram fresh tissue) was much less than that of glucose (average = 2.2 mg per gram tissue) from incubation mixtures containing an initial sugar concentration of 1 mg per ml. This suggests that 6FG equilibrated only with the extracellular space of the diaphragm in a manner similar to that observed with other sugars that are not metabolized[16].

Experiments with notatin (glucose oxidase) showed that 6FG was oxidized slowly by this enzyme; at 0.5 *M* concentration the rate of oxidation of 6FG was about 3% of that of glucose. This result is in harmony with the observations of KEILIN AND HARTREE that substitution in the 6-position of glucose reduces markedly its rate of oxidation by notatin[17].

In limited toxicity tests, 3 rats given single intraperitoneal injections of 250 mg of 6FG per kg of body weight showed toxic symptoms from which they recovered; similar doses administered on successive days resulted in the death of one rat after two doses and another rat after three doses. Thus 6FG is not highly toxic to rats.

Measurement of the possible effect of 6FG on a tissue culture capable of supporting synthesis of poliomyelitis virus[18] was made through the cooperation of Dr. H. E. ROBERTSON of the Department of Bacteriology, University of Minnesota. The results showed that addition of 6FG at concentrations as high as 1.2 mg per ml to a medium containing 1 mg of glucose per ml did not measurably influence the morphology of the uninfected cultures over an eight-day period or the rate and capacity of the cells to produce virus over a 40-hour period.

DISCUSSION

The marked inhibitory effect of 6FG on fermentation of glucose or of fructose by intact yeast in contrast to the small effect on fermentation by cell-free yeast extract gives evidence that the rate limiting step of fermentation in intact yeast differs from that in yeast extract. Support for this suggestion may also be deduced from the relative effect of substrate concentration on fermentation rate of intact and cell-free preparations. With intact yeast the rate of fermentation as measured manometrically is readily decreased by a decrease in glucose or fructose concentration (*e.g.* see Fig. 1 and ref.[12]); with a similar range of substrate concentrations the fermentation rate of yeast extract can not

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be limited. These results may be explained by the postulate that the fermentation rate by intact yeast is limited by the rate of transfer of substrate into the yeast cell, and that the rate of this transfer is decreased by addition of 6FG or decrease in the glucose or fructose concentration.

The similarity of the apparent K_i values for 6FG obtained with the same yeast preparation with either glucose or fructose as the substrate is in harmony with the suggestion that both glucose and fructose enter the cell by the same mechanism.

Postulates have been made that sugars may be phosphorylated in the process of transfer across cell membranes (see review by ROSENBERG AND WILBRANDT[19]). The activity of hexokinase does not appear to have been the rate limiting factor in the fermentation of glucose and fructose in the present studies for two reasons. First, as noted by others[14,20] and checked by us in connection with the present studies, the reduction of glucose or fructose concentrations required to decrease the activity of hexokinase is much greater than that required to reduce the rate of fermentation of these substrates by intact cells. Secondly, the inhibition by 6FG of the activity of yeast hexokinase with glucose or fructose as the substrate is much smaller than the inhibition of fermentation of the same substrates by intact yeast.

The existence of a specific transport mechanism for entry of glucose or fructose into the yeast cell as postulated herein has also been suggested by others[19], including ROTHSTEIN AND MEIER from studies of the effect of uranyl compounds[21], and by CRAMER AND WOODWARD[22] from studies on the inhibition of fermentation by 2-dexoxy-D-glucose.

SUMMARY

6-Deoxy-6-fluoro-D-glucose (6FG), at molar concentrations comparable to that of glucose or fructose present, produced a marked inhibition of the rate of fermentation of intact yeast. In contrast, 6FG has a much smaller inhibitory effect on the rate of fermentation by a cell-free yeast extract or on the activity of yeast hexokinase. The inhibition by 6FG of intact yeast fermentation was competitive with glucose or fructose, and was overcome by increasing the concentration of these substrates.

The rate of fermentation of glucose or of fructose by intact yeast was decreased by reduction in substrate concentration in a concentration range where the rate of fermentation by a yeast extract or the activity of hexokinase was unaffected.

These results support the postulate that 6FG inhibits a specific process, not limited by hexokinase activity, which controls the rate of entry of glucose and of fructose into the yeast cell.

The uptake of glucose by rat diaphragm was much less sensitive to the presence of 6FG than was fermentation of glucose by intact yeast. Notatin oxidized 6FG at a rate about 3% of that observed with glucose. 6FG was only moderately toxic to rats and did not affect the behavior of or virus synthesis in a tissue culture preparation.

RÉSUMÉ

Le 6-déoxy-6-fluoro-D-glucose (6FG), à des concentrations moléculaires comparables à celles du glucose ou du fructose présents, diminue fortement la vitesse de fermentation de la levure intacte. Au contraire, le 6FG a une action inhibitrice beaucoup moins marquée sur la fermentation d'un extrait de levure acellulaire ou sur l'activité de l'hexokinase de la levure. L'inhibition par le 6FG de la fermentation par la levure intacte est compétitive vis à vis du glucose ou du fructose, et est supprimée lorsqu'on augmente la concentration de ces substrats.

La vitesse de fermentation du glucose ou du fructose par la levure intacte diminue quand la concentration en substrat diminue dans un domaine de concentrations pour lequel la vitesse de fermentation d'un extrait de levure ou l'activité de l'hexokinase restent inchangées.

Ces résultats permettent de supposer que le 6FG inhibe un processus spécifique, non limité par l'activité de l'hexokinase, qui contrôle la vitesse de pénétration du glucose et du fructose dans la cellule de levure.

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La consommation du glucose par le diaphragme du rat est beaucoup moins sensible à la présence du 6FG que la fermentation du glucose par la levure intacte. La vitesse d'oxydation par la notatine est environ 3% de celle observée avec le glucose. La toxicité du 6FG pour le rat est modérée et n'influe pas sur le comportement ou la synthèse d'un virus dans une culture de tissu.

ZUSAMMENFASSUNG

6-Deoxy-6-Fluoro-D-Glucose (6FG) bewirkt bei einer Molkonzentration, die der der anwesenden Glucose oder Fructose gleicht, eine merkliche Hemmung der Gärungsaktivität ganzer Hefezellen. Dagegen war die Hemmwirkung von 6FG auf die Gärungsgeschwindigkeit von zellfreiem Hefeextrakt und auf die Wirksamkeit von Hefehexokinase viel geringer. In seiner Hemmwirkung auf die Gärungsaktivität ganzer Hefezellen konkurrierte 6FG mit Glucose und Fructose, so dass durch Steigerung der Konzentration dieser beiden Substrate die Hemmung rückgängig gemacht werden konnte.

Die Gärungsgeschwindigkeit von Glucose oder Fructose durch ganze Hefezellen konnte mittels Verminderung der Substratkonzentration in einem Konzentrationsbereich gehemmt werden, in dem die Gärungsgeschwindigkeit durch einen Hefeextrakt und die Hexokinasewirkung völlig unempfindlich blieben.

Diese Ergebnisse bestätigen die Vermutung, derzufolge durch 6FG ein spezifischer Vorgang, dessen Geschwindigkeit nicht durch Hexokinaseaktivität mitbestimmt wird, der aber die Aufnahme von Glucose und Fructose durch die Hefezellen regelt, gehemmt wird.

Die Aufnahme von Glucose durch Rattenzwerchfell war gegenüber der Anwesenheit von 6-FG viel unempfindlicher, als die Gärungsvorgänge der ganzen Hefezelle. Mit Notatin wurde eine Oxydation, die etwa 3% der Glucoscoxydation betrug, festgestellt. 6FG hat eine für Ratten nur geringe toxische Wirkung und beeinflusste die Funktion oder Synthese von Viren in einer Gewebszucht keineswegs.

REFERENCES

1 E. McDONALD AND F. B. CRAMER, *J. Franklin Inst.*, 253 (1952) 351.
2 E. McDONALD, *J. Franklin Inst.*, 257 (1954) 411.
3 R. PETERS, *Endeavour*, 13 (1954) 147.
4 J. D. JUDAH AND K. R. REES, *Biochem. J.*, 57 (1954) 374.
5 B. HELFERICH AND A. GNÜCHTEL, *Ber.*, 74 (1941) 1035.
6 B. HELFERICH AND M. VOCK, *Ber.*, 74 (1941) 1807.
7 B. HELFERICH, S. GRÜNLER AND A. GNÜCHTEL, *Z. physiol. Chem.*, 248 (1937) 85.
8 E. R. BLAKLEY, *Ph. D. Thesis*, University of Minnesota (1954).
9 H. E. ROBERTSON AND P. D. BOYER, *J. Biol. Chem.*, in press.
10 R. M. HOCHSTER AND J. H. QUASTEL, *Arch. Biochem. Biophys.*, 31 (1951) 278.
11 L. BERGER, M. W. SLEIN, S. P. COLOWICK AND C. F. CORI, *J. Gen. Physiol.*, 29 (1946) 379.
12 R. H. HOPKINS AND R. H. ROBERTS, *Biochem. J.*, 29 (1935) 919.
13 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
14 M. W. SLEIN, G. T. CORI AND C. F. CORI, *J. Biol. Chem.*, 186 (1950) 763.
15 W. C. STADIE AND J. A. ZAPP, JR., *J. Biol. Chem.*, 170 (1947) 55.
16 D. HAFT, I. A. MIRSKY AND G. PERISUTTI, *Proc. Soc. Exptl. Biol. Med.*, 82 (1953) 60.
17 D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 42 (1948) 221.
18 G. E. GIFFORD, H. E. ROBERTSON AND J. T. SYVERTON, *Proc. Soc. Exptl. Biol. Med.*, 86 (1954) 515.
19 T. ROSENBERG AND W. WILBRANDT, *Int. Review Cytology*, 1 (1952) 65.
20 V. D. WIEBELHAUS AND H. A. LARDY, *Arch. Biochem.*, 21 (1949) 321.
21 A. ROTHSTEIN AND R. MEIER, *J. Cellular Comp. Physiol.*, 32 (1948) 77.
22 F. B. CRAMER AND G. E. WOODWARD, *J. Franklin Inst.*, 253 (1953) 354.

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