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STUDIES ON INTESTINAL SUCRASE AND INTESTINAL SUGAR TRANSPORT

VI*. LIBERATION OF α-GLUCOSE BY SUCRASE AND ISOMALTASE FROM THE GLYCONE MOIETY OF THE SUBSTRATES

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SUMMARY

1. Sucrase–isomaltase isolated from rabbit intestine does not have detectable mutarotase activity.

2. The glucose which is liberated by either sucrase or isomaltase from the glycone moiety of the substrates retains the α-configuration of C-1.

3. The procedure developed to obtain these results involves freeze-drying of the samples, silylation and gas chromatographic separation.

INTRODUCTION

Some carbohydrases produce inversion of the configuration of the carbonyl carbon of the glycone moiety whereas others do not. Accordingly, different reaction mechanisms have been proposed (for review, see ref. 1).

Since intestinal sucrase–isomaltase has been isolated recently[2,3] it has now become possible to investigate whether these glucosidases liberate α- or β-glucose thereby distinguishing among possible mechanisms.

Prior to this investigation it was necessary to test our enzyme preparation for mutarotase activity. This problem also has an inherent interest since sucrase and sugar carrier are closely linked building blocks of the intestinal brush border membrane[4,5] and since the sugar carrier may have mutarotase activity[6–8]. It is therefore possible, *a priori*, that the sugar carrier, with its postulated mutarotase activity, is a part of the sucrase–isomaltase complex.

In the present investigation polarimetry was not thought to be the method of

* V: ref. 3.

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choice both because of its relatively low sensitivity and of the transglucosidase activity of sucrase[9] which produces oligosaccharides of unknown rotary power.

We have therefore applied gas chromatographic separation of the reaction products after conversion to their *O*-trimethylsilyl derivatives. It appears that gas chromatography has not been used previously in the determination of mutarotation or of the α, β configuration of reaction products. Independently, NEUKOM (unpublished results) is also using this technique in the study of the α, β configuration of sugars produced in the hydrolysis of polyuronic acids.

We have found that isolated sucrase–isomaltase has no mutarotase activity and that both sucrase and isomaltase liberate glucose in the α form.

The silylation of fructose can lead to six derivatives. Their identification is presently under investigation and some data have been already presented by one of us (C.-H. C.) in a preliminary form[10].

MATERIALS AND METHODS

Chemicals

Palatinose was a generous gift from Professor R. WEIDENHAGEN (Neuoffstein/Pfalz, W. Germany). It was freed from glucose by oxidation with glucose oxidase followed by filtration through DEAE-cellulose[11]. Hexamethyldisilazane and trimethylchlorosilane (purum) and α-glucose were purchased from Fluka AG, Buchs, Switzerland. All other reagents were of analytical grade.

Sucrase–isomaltase was isolated from rabbit intestine by a method already outlined[2,3]. It had a specific activity of 14 units/mg protein.

Silylation and chromatography

The sugars produced in the incubation mixtures (see below) were converted to their *O*-trimethylsylyl derivatives at room temperature, for the times given, essentially according to BENTLEY *et al.*[12]. The silylation mixture was composed of hexamethyldisilazane (1.0 ml), trimethylchlorosilane (0.5 ml) and anhydrous pyridine (8.5 ml). For chromatography, the following instruments were used: "F-20" gas chromatograph (Perkin–Elmer) with flame ionisation detector, and Aerograph 1520 gas chromatograph with double-column system and temperature programming. The conditions were: Silicon column: SE-30 3% on Gaschrom P, 80–100 mesh, diameter 2.7 mm, height 2 m. Column temperature (t_c): 152°. Injector temperature (t_j): 255°. Nitrogen: 50 ml/min. Programme: t_c = 152°, gradient 2°/min. 9 min 3 sec, gradient 4°/min up to 220° isotherm. Ethylene glycol succinate column: EGS 15% on Chromosorb W, 80–100 mesh, diameter 2.7 mm, height 2 m. t_c: 155°. t_j: 250°. Nitrogen: 50 ml/min, isotherm.

The results were calculated by comparing the area of each peak with the area of the peak of the standard (see Fig. 1).

Incubation conditions

All incubations were carried out at 37° in the absence of buffer because salts are known to increase the rate of spontaneous mutarotation (see *e.g.* ref. 11). The pH of the incubation mixtures was approx. 6 and was, therefore, not far from the

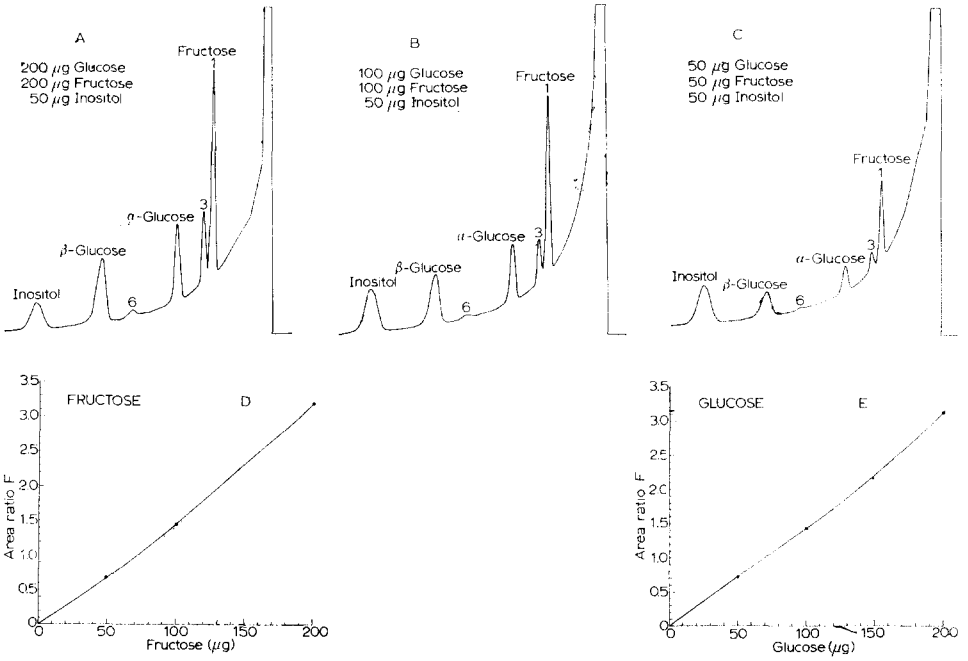


Fig. 1. Standard curve for glucose and fructose (equilibrium mixtures). A, B, C: gas chromatograms on ethylene glycol succinate (15%) of various amount of monosaccharides with 50 μg of inositol (internal standard). Peaks 1, 3 and 6 are various forms of fructose. D, E: standard curves for fructose, and glucose, respectively, from chromatograms A, B, and C. On the ordinates, the ratio (F) between the area of the fructose (or glucose) peak and the area of the internal standard (inositol) is plotted.

pH optimum of sucrase activity[3,9,13] and from that pH where glucose has the minimum spontaneous mutarotation rate, namely, 4.61 (ref. 14).

Mutarotation of glucose

Determination of the velocity constants of the spontaneous mutarotation. The incubation mixtures (0.5 ml) contained 0.1 mg of α-glucose each (the solution was prepared immediately before use from a crystalline preparation of α-glucose which contained only a very few percent of the β form; water prehaeted at 37° was used). At 15 sec, 1, 2, 3, 9 and 12 min the samples were frozen by immersing the incubation tube in liquid air; they were lyophilized, silylated with 0.05 ml of silylation mixture for 30 min and chromatographed on the Silicon column.

The velocity constants $\left(\alpha \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \beta\right)$ were calculated from the composition of the equilibrium mixture (α-glucose, 37.9%; β-glucose, 62.1% (Fig. 2, F), and from the equation

$$k_1 + k_{-1} = \frac{2.303}{t_2 - t_1} \log \frac{\beta_{eq} - \beta_1}{\beta_{eq} - \beta_2} \qquad (1)$$

where β_{eq}, β_1 and β_2 are the concentrations of β-glucose at equilibrium, and at the times t_1 and t_2, respectively. In the experiment reported (Fig. 2) t_1 was 15 sec; the sample taken at this time contained 3.65% of the β form.

The constants obtained were: $k_1 = 0.0525$ and $k_{-1} = 0.0320$ min^{-1}. $K = k_1/k_{-1} = 1.64$. They correlate well with data in the literature, if allowance is made for the different temperature (Q_{10}, about 2.5) and the different logarithms used[14].

Test for possible mutarotase activity in sucrase–isomaltase

The incubation mixtures (1 ml) contained glucose (1 mg; the solution was prepared immediately before use from crystalline α-glucose, which was found to contain some 23% of the β form) and 0.076 sucrase units of pure sucrase-isomaltase. Controls were run without enzyme. At 0, 3 and 6 min the samples were frozen with liquid air, as above, lyophilized, stored in a desiccator over P_2O_5 for not more than a few hours, silylated with 0.5 ml of silylation mixture for 2 h, and chromatographed.

Determination of the sugars formed during hydrolysis of sucrose

The incubation mixtures (0.5 ml) contained sucrose (15 μmoles) and sucrase (0.74 sucrase units). At 2, 6, and 20 min the samples were frozen as above; the internal standard (inositol, 50 μg in 0.1 ml water) was added on top of the frozen samples, and frozen; the whole sample was lyophilized and silylated with 1.2 ml of silylation mixture overnight. 1 ml of the reaction mixture was concentrated *in vacuo* and again treated with 0.2 ml of silylation mixture for 2 h and chromatographed on both columns.

Determination of the sugars formed during hydrolysis of palatinose

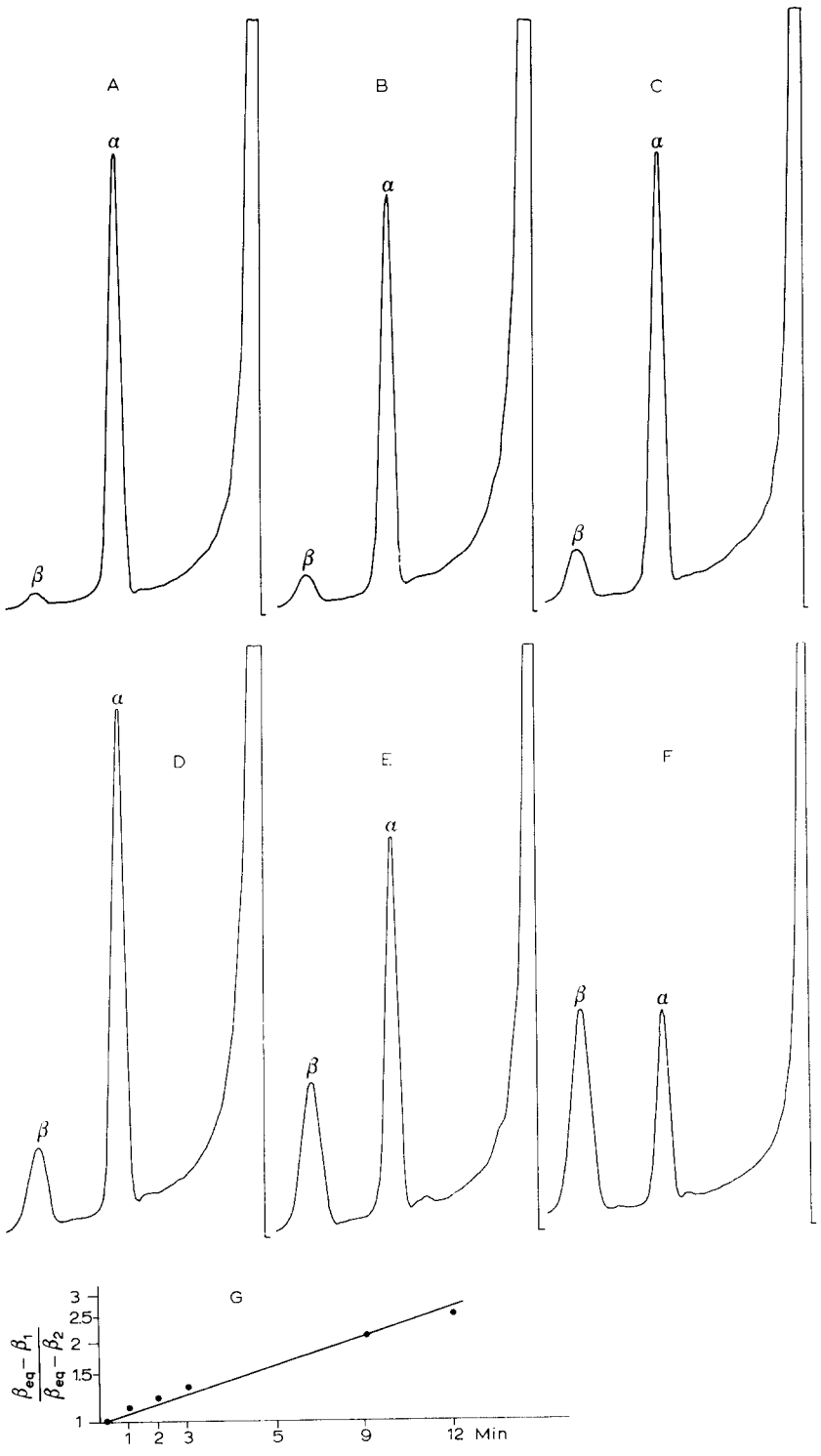
As a substrate for isomaltase, palatinose (isomaltulose) was used since it is split at the same site as isomaltose[3], but, unlike isomaltose, it liberates glucose from the glycone moiety alone. The incubation mixtures (2 ml) contained palatinose (100 μmoles) and sucrase–isomaltase (0.265 sucrase units). The samples were processed as for sucrose hydrolysis (see above), except that the first silylation was carried out in 5.6 ml of silylation mixture.

RESULTS AND DISCUSSION

The gas-chromatographic separation of the silyl derivatives of glucose and fructose

Under the conditions applied[12] silylation proceeds much faster than the spontaneous mutarotation of glucose in anhydrous pyridine: almost no β-glucose could be detected if crystalline α-glucose was dissolved directly into the silylation mixture. This agrees with what is reported in the literature for glucose and for other sugars[12,15]. During lyophilization some mutarotation does occur, but even so the total error hardly accounts for more than a few percent β-glucose. In fact, the sample of α-glucose incubated for 15 sec at 37° (Fig. 2), and subsequently frozen-dried and silylated, yielded 3.6% of the β form. Of this, 1.36% is accounted for by the spontaneous mutarotation occurring during the 15 sec incubation (see above the velocity constants under these conditions). This leaves us with a maximum of 2.24% β-glucose formed during lyophilization.

Fructose, either crystalline or from a frozen-dried solution, yields on ethylene glycol succinate columns a much more complex pattern, which depends in part on how long silylation is allowed to proceed. The peaks numbered 1 and 3 in Fig. 4 have been identified by infrared and mass spectrometry as persilylated cyclic deriva-


A
B
C
α
β
D
E
F
G
$\frac{\beta_{eq}-\beta_1}{\beta_{eq}-\beta_2}$
3
2.5
2
1.5
1
1 2 3 5 9 12 Min

tives*. Peak 6 carries a free carbonyl group and presumably corresponds to the persilylated linear form. Complete identification of these peaks and of those arising at earlier silylation times is presently in progress*.

The use of gas chromatography for the determination of mutarotase activity and of the configuration of products of carbohydrases

Mutarotation rates as well as the configuration of the carbonyl carbon of sugars have usually been determined polarimetrically. Enzymes have also been used for the same purposes, because of their high specificity for either configuration of the carbonyl carbon[16]. The procedure developed in the present paper—freeze-drying followed by gas-chromatographic separation of the silyl derivatives—has some advantages over polarimetry (it is more sensitive and it allows analysis of individual components in complex reaction mixtures), as well as over enzymatic procedures (it does not require the time-consuming preparation of a purified enzyme and it can be applied to sugars for which no appropriate enzyme is available). Furthermore, since the chromatographic retention time decreases approximately according to the increasing number of axial substituents[15,17], it may provide a further indication on the configuration of the carbonyl carbon. Finally, individual chromatographic peaks can be trapped for further analysis (see NOTE ADDED IN PROOF).

Lack of mutarotase activity in sucrase–isomaltase

Fig. 3 shows that the presence of sucrase–isomaltase does not increase the mutarotation rate of glucose over the spontaneous rate. Also, this lack of mutarotase

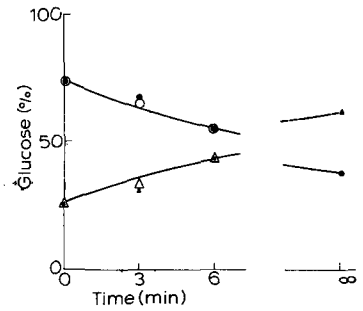


Fig. 3. Lack of mutarotase activity in sucrase–isomaltase. Percent of β-glucose in the presence (△) and in the absence (▲) of sucrase–isomaltase; percent of α-glucose in the presence (○) and in the absence (●) of sucrase–isomaltase.

activity is demonstrated by the agreement between the observed $\alpha/(\alpha + \beta)$ ratio for the glucose formed during the hydrolysis of sucrose and the ratio calculated

* H. C. CURTIUS, W. SIMON, M. MÜLLER AND G. SEMENZA, unpublished results.

Fig. 2. Spontaneous mutarotation of α-glucose at 37°. Conditions, see under METHODS. A, after 15 sec; B, after 1 min; C, after 3 min; D, after 9 min; E, after 12 min; F. equilibrium mixture; G, semilogarithmic plot according to Eqn. 1. The regression line calculated from the points of G gives a value for $(k_1 + k_{-1})$ of 0.0845 min^{-1}, with a standard deviation of 0.004260, and limits of confidence for $P = 95\%$ of $0.07266 < (k_1 + k_{-1}) < 0.09632$ min^{-1}.

TABLE I

MONOSACCHARIDES LIBERATED DURING THE HYDROLYSIS OF SUCROSE BY INTESTINAL SUCRASE

The ratio $\alpha/(\alpha + \beta)$ was calculated from Eqn. 2 and the velocity constants for spontaneous mutarotation of glucose. For details, see under *Incubation conditions*.

Time (min)	*Glucose (μg)*					*Total fructose (μg)*
	α	β	$\alpha + \beta$	$\alpha/(\alpha + \beta)$ *Found*	$\alpha/(\alpha + \beta)$ *Calculated (Eqn. 2)*	
2	33.3	3.7	37	0.90	0.948	33
6	85.1	12.9	98	0.868	0.870	70
20	283.8	114.2	398	0.71	0.684	456

from certain assumptions, including the lack of mutarotase activity in the enzyme used (see below, Table I).

As pointed out in the introduction, sucrase–isomaltase was tested for mutarotase activity in connection with the possible mutarotase activity of the carrier for sugars[6–8] and with respect to the vicinity of sucrase and sugar carrier[3,4]. Obviously, the lack of mutarotase activity in sucrase–isomaltase is not necessarily against the suggested identity of mutarotase and sugar carrier.

The configuration of glucose liberated during the hydrolysis of sucrose and of palatinose (Fig. 4 and Tables I and II)

At all times considered, more α-glucose is present than β-glucose. Since the

TABLE II

GLUCOSE LIBERATED DURING THE HYDROLYSIS OF PALATINOSE BY ISOMALTASE

Chromatographic column	*α-Glucose (μg)*			*β-Glucose (μg)*		
	Silicon	*Ethylene glycol succinate*	*Average*	*Silicon*	*Ethylene glycol succinate*	*Average*
Time						
2 min 37 sec	8	9	8.5	7	6	6.5
6 min 37 sec	39	35	37	14	13	13.5

equilibrium is in favour of the β form, it is obvious that sucrase and isomaltase (which hydrolyses palatinose[3]) liberate glucose mainly or exclusively as α-glucose.

This qualitative argument is further supported by the following quantitative considerations. If one assumes (a) that α-glucose *alone* (besides fructose) arises during hydrolysis of these disaccharides and that β-glucose arises from α-glucose by spontaneous mutarotation only, (b) that the enzymatic hydrolysis of these disaccharides follows zero-order kinetics under the conditions applied, as indicated by the data of Table I (column "$\alpha + \beta$"), and (c) that transglucosidation is negligibly

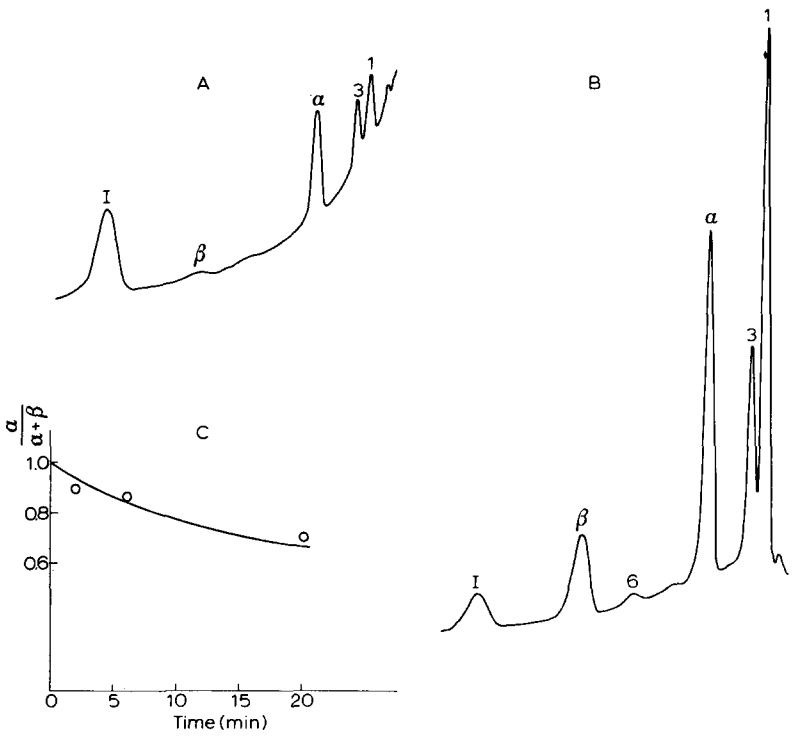


Fig. 4. Monosaccharides formed during the enzymatic hydrolysis of sucrose at 37°. 1, 3, 6: fructose peaks; α, α-glucose; β, β-glucose; I, internal standard (inositol). A, after 2 min incubation; B, after 20 min incubation; C, comparison between the $\alpha/(\alpha+\beta)$ ratio found (○) and the ratio calculated from Eqn. 2 (continuous line). Chromatographic column: ethylene glycol succinate.

small compared to hydrolysis, then the ratio α-glucose to total glucose $(\alpha/(\alpha+\beta))$ at the time t is given by the equation*:

$$\frac{\alpha}{\alpha+\beta} = \frac{1}{K+1} + \frac{1}{k_1 t}\left(\frac{K}{K+1}\right)^2\left(1 - e^{-(k_1 + k_{-1})t}\right) \qquad (2)$$

In the calculations (Table I) we used the values of the equilibrium constant ($K = k_1/k_{-1}$) and of the velocity constants k_1 and k_{-1} for the *spontaneous* mutarotation of glucose. The comparison with the $\alpha/(\alpha+\beta)$ ratio actually found (Fig. 4, C and Table I) conclusively shows that no (or only traces of) β-glucose is formed by sucrase.

Intestinal sucrase and isomaltase, therefore, belong to the group of glycosidases which do not produce inversion of the carbonyl carbon, together with α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1)[18], *Escherichia coli* β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23; ref. 16) and others.

The present data render unlikely a single simple S_{N_2} reaction taking place at the C-1 of the glucosyl moiety of sucrose or isomaltose. They are compatible, however, with a number of other mechanisms, including double displacement[1,19] and front-side displacement[1].

Finally, the observation that glucose is liberated in the α form during the enzymatic hydrolysis has a practical interest. Since glucose oxidase oxidizes the β form

* We thank Professor F. LEUTHARDT for deriving Eqn. 2.

only[20,21], our data provide a likely explanation for the time lag observed when disaccharidases are coupled with glucose oxidase[11,21].

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REFERENCES

1 D. E. KOSHLAND, JR., in W. D. MCELROY AND B. GLASS, *The Mechanism of Enzyme Action*, Baltimore, Md., 1954, p. 608.
2 J. KOLÍNSKÁ AND G. SEMENZA, *Helv. Physiol. Acta* ,24 (1966) C 30.
3 J. KOLÍNSKÁ AND G. SEMENZA, *Biochim. Biophys. Acta*, 146 (1967) 181.
4 D. MILLER AND R. K. CRANE, *Biochim. Biophys. Acta*, 52 (1961) 281.
5 D. MILLER AND R. K. CRANE, *Biochim. Biophys. Acta*, 52 (1961) 293.
6 A. S. KESTON, *Science*, 120 (1954) 355.
7 A. S. KESTON, *Science*, 143 (1964) 698.
8 J. M. BAILEY, P. G. PENTCHEV AND J. WOO, *Biochim. Biophys. Acta*, 94 (1965) 124.
9 A. DAHLQVIST, *Acta Chem. Scand.*, 14 (1960) 63.
10 C.-H. CURTIUS, *Varian-Aerograph Gas Chromatography*, 1966, Abstracts, p. 3.
11 M. MESSER AND A. DAHLQVIST, *Anal. Biochem.*, 14 (1966) 376.
12 R. BENTLEY, C. C. SWEELEY, M. MAKITA AND W. W. WELLS, *Biochem. Biophys. Res. Commun.*, 11 (1963) 14.
13 J. A. CARNIE AND J. W. PORTEOUS, *Biochem. J.*, 85 (1962) 450.
14 H. S. ISBELL AND W. W. PIGMAN, *J. Res. Natl. Bur. Std.*, 20 (1938) 773.
15 W. W. WELLS, C. C. SWEELEY AND R. BENTLEY, in H. A. SZYMANSKI, *Biomedical Applications of Gas Chromatography*, Plenum Press, New York, 1964, p. 169.
16 K. WALLENFELS AND G. KURZ, *Biochem. Z.*, 335 (1962) 559.
17 R. J. FERRIER, *Tetrahedron*, 18 (1962) 1149.
18 R. KUHN, *Ann. Chemie*, 443 (1925) 1.
19 K. WALLENFELS AND O. P. MALHOTRA, *Advan. Carbohydrate Chem.*, 16 (1961) 239.
20 R. BENTLEY AND A. NEUBERGER, *Biochem. J.*, 45 (1949) 584.
21 D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 50 (1952) 331.