The Molecular Structure and Shape of Yeast Glycogen

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Since the recognition of glycogen in yeast by Errera (1885) there have been a number of attempts to isolate it and to compare its nature with that of various animal glycogens. The earlier work used optical rotation, opalescence of aqueous solution, colour with iodine and differences in mineral, phosphorus and nitrogen contents of the samples, as a basis for comparison. Jeanloz (1944) pointed out that comparisons of this nature were unsuitable for detecting possible differences among glycogens and he extended the studies by fractionation of the glycogen by electrodialysis, viscosity determinations of the triacetate, and measurement of the percentage hydrolysis brought about by wheat β-amylase.

In the work to be described an attempt has been made to obtain more precise information concerning the structure of yeast glycogen. Methylation and periodate oxidation were used to determine the type of linkage of the glucose radicals and the average chain length. The position of the branching point in the unit chain was found by a study of the extent of hydrolysis with pure crystalline β-amylase. A comparison of this amylolysis limit with that produced by a non-crystalline β-amylase, containing a trace of Z enzyme, was used to indicate the probable absence of β-1:3 links in the chains. In addition, some idea of the shape of the glycogen molecule has been obtained by viscosity measurements. A comparison of samples of the glycogen prepared by different methods of extraction has also made it possible to offer an explanation of the findings of various workers which seemed to indicate the presence of at least two chemically and functionally different glycogens in the yeast cell (Ling, Nanji & Panton, 1925; McAnally & Smedley-Maclean, 1937; Trevelyan & Harrison, 1952).

EXPERIMENTAL AND RESULTS

Methods and materials

The yeast, general analyses for N and P, and the paper-chromatographic methods used for the sugars were the same as those described previously (Northcote & Horne, 1952). The paper chromatograms of the methylated glucose derivatives were run for 12 hr. at room temperature. The rabbit-liver glycogen was kindly given by Dr D. J. Bell; it had a chain length of 12 by periodate oxidation (Bell & Manners, 1951).

Isolation of the glycogen

Three methods were adopted based on preparations previously described in the literature. In each case 36 g. of the same sample of pressed baker's yeast were used.

(A) *Cytolysis with* 3% NaOH. The method of Bell & Northcote (1950) was used followed by extraction of the glycogen from the insoluble residue and the alkaline solution.

(i) Extraction from the insoluble residue. This was heated at 75° with 700 ml. 0·5N-acetic acid for 2 hr.; pH of the mixture approx. 2. The mixture was centrifuged and the solid washed with water at 75° and recentrifuged. The washings were repeated until the supernatant gave no precipitate with ethanol or colour with I_2. The extract and washings were combined and precipitated with ethanol. The glycogen obtained was purified, until its N content was less than 0·1%, by solution in water and precipitation with ethanol.

(ii) Extraction from the alkaline solution. The solution was made just acid with acetic acid and any insoluble material removed by centrifugation. The supernatant was

Table 1. *Glycogen prepared by three methods from baker's yeast*

(The ash, N and P contents of all the samples of glycogen were less than 0·1%. The glycogen was dried at 100° over P_2O_5 at 0·01 mm. Hg for 6 hr. in each case.)

	Glycogen extracted from insoluble residue (i)		Glycogen in solution (ii)	
	Yield (% dry wt.)	$[\alpha]_D^{18°}$ in water	Yield (% dry wt.)	$[\alpha]_D^{18°}$ in water
(A) NaOH-cytolysed pressed yeast	3·2	+184° (*c*, 0·76; *l*, 2 dm.)	0·0	—
(B) NaOH-cytolysed dried yeast	2·0	+184° (*c*, 0·5; *l*, 2 dm.)	0·8	+186° (*c*, 0·8; *l*, 2 dm.)
(C) Mechanically broken yeast	0·0	—	1·5	+188° (*c*, 1·1; *l*, 2 dm.)

evaporated under reduced pressure at 60° to 20 ml. and the polysaccharides precipitated with ethanol. The subsequent purification of the glycogen followed the method of Daoud & Ling (1931).

(B) *Use of dried yeast.* The drying was carried out at 15° for 48 hr. in a vacuum desiccator (0·01 mm. Hg) over P_2O_5. The procedures were then those described for A (i) and A (ii).

(C) *Cytolysis by grinding with fine sand.* The method of Harden & Young (1912) was used. The grinding was thorough and maintained for 45 min. and the mass poured directly into boiling water (500 ml.) and extracted for 2 hr. The solution was decanted and the residue re-extracted with 250 ml. of boiling water. The solution and the washings were combined and the glycogen subsequently isolated from the solution and the insoluble residue by the procedures described for A (i) and A (ii).

The yields and characteristics of the material obtained by these methods are given in Table 1.

Acid hydrolysis of the glycogen

Each sample of glycogen (100 mg.) was hydrolysed for 3 hr. on a boiling-water bath with 10 ml. N-HCl. The hydrolysates were neutralized with Ag_2O and the excess Ag removed by H_2S in the usual manner. The resultant solutions were clarified by charcoal, concentrated under reduced pressure to 5 ml. and investigated separately on paper chromatograms. Glucose was the only sugar found. The glucose content of each sample of glycogen was estimated by the anthrone method as used by Seifter, Dayton, Novic & Muntwyler (1950); it was never less than 98·5%.

Methylation of the glycogen

The glycogen (A (i), 5 g.) was first acetylated then simultaneously deacetylated and methylated according to the method of Haworth & Percival (1932). Eight successive methylations were performed and the solid material was finally extracted with boiling $CHCl_3$, dried over anhyd. $MgSO_4$, concentrated under reduced pressure and the methylated polysaccharide precipitated by light petroleum, b.p. 40–60°.

The yield of acetylated glycogen was 7·4 g. (83% of theory). $[\alpha]_D^{17°}$ +158° in $CHCl_3$ (*c*, 1·0; *l*, 2 dm.). (Found: CH_3CO, 44·5%.) The yield of methylated glycogen was 4·4 g. (83·5% from 7·4 g. of the acetate). $[\alpha]_D^{17°}$ +200° in $CHCl_3$ (*c*, 1·0; *l*, 2). (Found: OMe, 45·0%.)

Hydrolysis of the methylated glycogen

The material (2·0 g.) was refluxed with 50 ml. of methanol containing 2% (w/w) HCl for 12 hr. After removal of the HCl by treatment with Ag_2CO_3 and removal of the solid by filtration, the resulting solution was evaporated under reduced pressure. The residual syrup, dissolved in N-HCl, was heated under reflux on a boiling-water bath for 12 hr. The solution was freed from HCl by an excess of Ag_2CO_3 in the customary manner and concentrated under reduced pressure to a syrup in the presence of $BaCO_3$ (Bell, 1944).

Identification and separation of the cleavage products

The solution was subjected to a chromatographic separation by the method of Bell (1944) using a 3 g. column of silica gel prepared according to Tristram (1946). Three fractions were obtained, two of which, the tetra- and trimethyl fractions, crystallized. Yield: Me_4, 0·180 g.; Me_3, 1·356 g.; Me_2, 0·417 g.; aggregate yield 89·5%. The average chain length calculated on the molecular ratios of methylated sugars recovered is 11·7 glucose units/non-reducing end group.

The tetramethyl fraction had m.p. 93°, not depressed on admixture of an authentic specimen of 2:3:4:6-tetramethyl glucose (m.p. 95°). $[\alpha]_D^{20°}$ +83°, equilibrium value in water (*c*, 1·8; *l*, 2 dm.). (Found: OMe, 51·5. Calc. for $C_{10}H_{20}O_6$: OMe, 52·5%.) On the paper chromatogram the whole fraction showed a single compact spot identical with a control sample of 2:3:4:6-tetramethyl glucose.

The whole trimethyl fraction had $[\alpha]_D^{18°}$ +90° in water, falling to +68° (*c*, 2·0; *l*, 2 dm.). The crystals melted at 108° and on admixture did not depress the melting point of authentic 2:3:6-trimethyl glucose (m.p. 113°).

The sample was recrystallized from ether. (Found: OMe 41·1. Calc. for $C_9H_{18}O_6$: OMe, 41·9%.) The rotation of the methylated sugar was depressed to a negative value by solution in methanol containing 2% (w/w) HCl. $[\alpha]_D^{18°}$ +68° initially; $[\alpha]_D^{18°}$ −28° after 24 hr. (*c*, 0·6; *l*, 2 dm.). Tne whole fraction without recrystallization showed a single compact spot on a paper chromatogram identical with authentic 2:3:6-trimethyl glucose.

The dimethyl fraction showed the presence of at least two components (i.e. two distinct spots) when it was investigated on a paper chromatogram. The probable identity of these was only inferred by comparing them on a paper chromatogram with 2:3- and 3:6-dimethyl glucoses when a correspondence was obtained; the R_G values were 0·55 and 0·48, respectively. It is possible that the presence of 3:6-dimethyl glucose in the hydrolysate results from incomplete methylation of the glycogen.

End-group assay of the glycogen by periodate oxidation

This was carried out by the method of Halsall, Hirst & Jones (1947). Glycogen (A (i), 91·2 mg.) was dissolved in 70 ml. of a solution containing 7 g. KCl and 20 ml. 8% (w/v) sodium metaperiodate solution. A control without glycogen was investigated simultaneously. The solutions were kept agitated on rollers. Samples (10 ml.) were taken at intervals, excess periodate decomposed by the addition of 0·5 ml. ethylene glycol (pH 5 to methyl red), and the formic acid present titrated against 0·01 N-NaOH in CO_2-free air with methyl red as indicator. The following results were obtained:

Time of sampling (hr.)	48	168	192
Titre (ml. 0·0104 N-NaOH)	0·46	0·64	0·64

The titre, 0·64 ml. of the alkali, is equivalent to 2·14 mg. of formic acid and corresponds to a unit chain length of 12 glucose radicals. The experiment was repeated using glycogen *c*, 378·7 mg. of which in 120 ml. of solution yielded 9·77 mg. of formic acid, corresponding to a chain length of 11·2 glucose units.

Hydrolysis of the glycogen by β-amylase

The enzyme was prepared by extracting barley flour (100 g.) with water (300 ml.) for 1 hr. The resulting solution after dialysis contained α- and β-amylases and maltase. α-Amylase activity was destroyed by storage at pH 3·6 and

4° for 1 week; β-amylase was isolated by ethanolic fractionation of the solution according to the method of Halsall, Hirst, Hough & Jones (1949).

The freeze-dried enzyme contained no maltase and failed to hydrolyse starch beyond 60% (possible maltose) on prolonged incubation, thus indicating the absence of α-amylase. Small amounts of Z enzyme (Peat, Pirt & Whelan, 1952*a*) were present, as shown by the hydrolysing action of this preparation on yeast glucan and on laminarin (Manners, 1952*a*).

Crystalline β-amylase (sweet potato) (Balls, Thompson & Walden, 1946; Balls, Walden & Thompson, 1948) was kindly given by Dr C. S. Hanes, F.R.S. This enzyme was free from Z-enzyme activity.

The action of β-amylase on rabbit-liver glycogen and yeast glycogen (A (i) and B (ii)) was studied. About 30 mg. of the polysaccharide, dissolved in 2 ml. of water, were treated with 5 ml. of 0·2 M-sodium acetate buffer (pH 4·6), and 3 ml. of enzyme solution (approx. 0·1 mg./ml.). This mixture was incubated at 25° under toluene and 2 ml. samples were withdrawn at intervals for determination of the reducing power by the method of Shaffer & Somogyi (1933) as modified by Hanes & Cattle (1938). The results obtained with β-amylase-containing Z enzyme are shown in Fig. 1. In all cases the enzyme was active at the end of the experiment, as shown by an increase in reducing power on the addition of a small amount of starch solution. Additional digests were set up using yeast glycogen with the following modifications:

(*a*) Previous incubation of the glycogen with emulsin (commercial B.D.H. preparation) (Peat, Thomas & Whelan, 1952).

(*b*) The non-crystalline β-amylase was preheated at 60° at pH 4·8 for 30 min. to destroy Z enzyme (Peat, Pirt & Whelan, 1952*b*).

(*c*) The buffer used was changed to pH 3·6 to inhibit the action of Z enzyme (Peat *et al.* 1952*a*).

In (*b*) and (*c*) three separate additions of enzyme after 96 hr. intervals were needed to reach the hydrolysis limit. The results for the above experiments are summarized in Table 2.

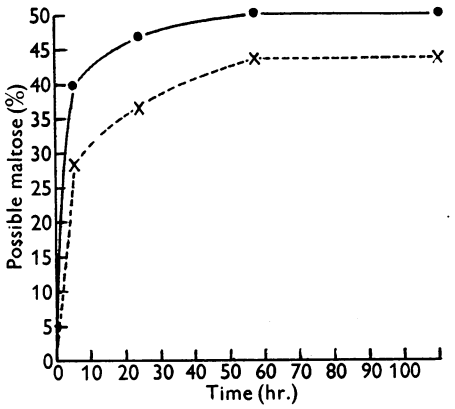

Fig. 1. Hydrolysis of yeast and rabbit-liver glycogens by barley β-amylase, pH 4·6; temp. 25°. ●—●, yeast glycogen A (i); ×- - -×, rabbit-liver glycogen.

The viscosities of yeast glycogen and rabbit-liver glycogen

The determinations were made at 21° in a horizontal capillary viscosimeter (Tsuda, 1928; Ostwald, 1933). The capillary had the following characteristics: radius, 0·0222 cm.; length, 28·60 cm.; flow volume, 1·128 ml. The yeast glycogen used was preparation A (i) and the concentrations of the aqueous solutions varied over the range 0·4–2·0%. Fig. 2 shows the results obtained at constant pressure (47·9 cm. of water). The viscosity of the yeast glycogen was also measured at varying pressures and it was found to be independent of the rate of shear (range investigated 290–2100 sec.$^{-1}$). Thus the flow of this solution is strictly Newtonian.

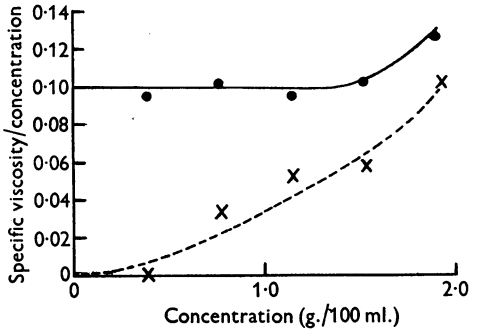

Fig. 2. Viscosities of yeast and rabbit-liver glycogens in aqueous solution at 21°. ●—●, yeast glycogen; ×- - -×, rabbit-liver glycogen.

Table 2. *Extent of hydrolysis of glycogen by β-amylase at 25°*

	Percentage possible maltose at hydrolysis limit		
	Yeast glycogen		Rabbit-liver glycogen
	A (i)	B (ii)	
Crystalline β-amylase, pH 4·6	49	50	43
Crystalline β-amylase + emulsin, pH 4·6	50	50	—
Barley β-amylase + Z enzyme, pH 4·6	50	50	43
Barley β-amylase + Z enzyme, pH 3·6	49	—	—
Barley β-amylase (after heating to 60°, pH 4·8)	50	—	—

The sorption of water vapour by rabbit-liver glycogen and yeast glycogen A (i)

The glycogens were dried at 100° over P_2O_5 at 0·01 mm. Hg to constant weight. Approximately 0·2 g. portions were allowed to come to equilibrium at 26° with water vapour in closed desiccators containing H_2SO_4 solutions of various known concentrations (Wilson, 1921). Equilibrium was reached with the low relative water-vapour-pressure systems in about 5 days; with high humidities about 9 days were necessary. The amount of water taken up by the glycogens was obtained by direct weighing, using well stoppered weighing bottles. In all cases the amount of water taken up by the two glycogens was very similar (Table 3). A detailed study of the moisture sorption isotherms of these glycogens will be reported separately.

Table 3. *The sorption of water vapour by yeast and rabbit-liver glycogens at 26°*

Relative humidity (%)	Water sorbed (g./100 g. dry wt. of glycogen)	
	Yeast glycogen	Rabbit-liver glycogen
5·0	2·9	3·1
38·5	9·7	9·9
68·0	14·9	15·2
91·0	32·2	31·2

DISCUSSION

The methods used for the extraction of yeast glycogen have been of two main types: (*a*) mechanical breakage of the cell by grinding with fine sand (Harden & Young, 1912; Stockhausen & Silbereisen, 1936; Jeanloz, 1944), (*b*) alkaline cytolysis using either dilute (2–3%) sodium hydroxide solution (Salkowski, 1894; Ling *et al.* 1925) or 30–60% sodium hydroxide solution (McAnally & Smedley-Maclean, 1937; Trevelyan & Harrison, 1952). Method (*b*) gives rise to some glycogen in solution and an insoluble residue containing glycogen; the latter can be rendered soluble by pretreatment with cold N-HCl (McAnally & Smedley-Maclean, 1937) or by acetic acid as in the present work (see also Bell & Northcote, 1950). Use of 3% sodium hydroxide solution on pressed baker's yeast failed to bring any of the glycogen into solution. Ling *et al.* (1925) used dried brewer's yeast and obtained glycogen in the dilute sodium hydroxide solution. It has been shown in the work described here that when the baker's yeast is dried some glycogen can be rendered soluble by treatment with dilute sodium hydroxide solution. It seems likely from the microscopical studies on the yeast cell wall preparations (Northcote & Horne, 1952) that all of the glycogen is freed from the cell wall by a complete mechanical breakage of the cell. It has been shown that some glycogen is rendered soluble in water by the incomplete breakage which is obtained by grinding yeast with sand. The insolubility of the glycogen in the sodium hydroxide preparations would appear to be due to an envelopment of the glycogen by the insoluble glucan membrane and therefore to be mechanical rather than chemical. Concentrated alkali and strong acids will subsequently affect the permeability of the membrane, allowing the glycogen to diffuse. If the glycogen is polydisperse (Bridgeman, 1942; Bell, Gutfreund, Cecil & Ogston, 1948; Record, 1948) it is not surprising that some of the molecules can pass into solution through the glucan membrane while others are held back. It is known that drying affects the permeability of the yeast membrane (Thorsell & Myrbäck, 1951) and thus a ready explanation is afforded for the solubility of the glycogen after the yeast has been dried.

The chemical analysis of the polysaccharide shows that for the most part it is very similar to rabbit-liver glycogen. Its chain length, as given by end-group assay of the methylated polysaccharide and periodate oxidation, is 11 or 12 glucose units. The relative amounts of tetra-, tri- and di-methylglucoses obtained after hydrolysis of the methylated product shows that the majority of the links within the glycogen are of the 1:4 type. The analysis resembles that obtained by Hirst, Hough & Jones (1949) for rabbit-liver glycogen and the similarity of the chromatograms of the dimethyl fractions probably indicates that the interchain links of the two glycogens are of the same type. The values of the specific rotations obtained for yeast glycogen and the acetylated and methylated products were of the order to be expected for a polysaccharide in which the glucosidic links are α, and this was further supported by the enzymic investigation discussed below. The hydrolysis of the yeast polysaccharide by β-amylase, which is specific for 1:α:4-glucosidic links, gives 50% of the possible maltose. Jeanloz (1944) has already reported a hydrolysis limit, using wheat amylase, of 48% on one of the fractions of yeast glycogen obtained by electrodialysis. These amylolysis limits are about 5% higher than for most glycogens (Manners, 1952*b*) and therefore show a slight difference in the position of the branching points of the chains from those of animal glycogens (Meyer & Fuld, 1941). Z enzyme and emulsin have no action on the glycogen and thus the anomalous links which occur in amyloses are probably absent. In both the chemical and enzymic work, when comparisons were made between the samples of the yeast glycogen prepared by the various methods, no differences could be detected, and thus there appears to be only one type of glycogen in the yeast cell. This glycogen functions as a normal storage

substance and not as a structural unit of the cell wall (Northcote & Horne, 1952).

Because of the apparent similarity between the chemical structure of rabbit-liver glycogen and yeast glycogen it is interesting to note the significant difference in their viscosities (Fig. 2). This difference indicates a more asymmetric nature of the yeast polysaccharide (the intrinsic viscosity of the liver glycogen is almost zero and hence this molecule is approximately spherical in shape), or a difference in the degree of hydration, or a combination of both of these factors. Some idea of the extent of hydration of the two polysaccharides in solution may be obtained from the moisture sorption experiments. These show a surprising degree of similarity and therefore may indicate a similarity in the degree of hydration in solution. The value of the intrinsic viscosity of the yeast glycogen calculated from the values shown in Fig. 2 is 0·102 (S.D. = 0·010). Since the flow is Newtonian an estimate of the axial ratio of the yeast glycogen molecule can be made from Simha's equation (Simha, 1940; Mehl, Oncley & Simha, 1940) taking the partial specific volume of the glycogen to be 0·65 (Bell *et al.* 1948) and correcting for hydration according to Oncley (1941). The ratio obtained is approximately 8:1 if the shape is considered as a prolate ellipsoid of revolution, or 1:13 as an oblate ellipsoid of revolution.

SUMMARY

1. Samples of yeast glycogen have been prepared by various methods. A comparison of the products has failed to give any evidence for the existence of more than one glycogen in yeast. An explanation is put forward to account for the insolubility of part of the yeast glycogen when the cell is cytolysed by sodium hydroxide solution.

2. Yeast glycogen has been shown to be composed of glucose molecules linked for the most part by 1:α:4 links in unit chains of approximately 12 glucose radicals.

3. The hydrolysis limits obtained by the action of β-amylase have indicated some slight difference in the position of the branching point in yeast glycogen and in rabbit-liver glycogen. No anomalous links hydrolysable by Z enzyme or emulsin have been found in yeast glycogen.

4. The viscosity measurements indicate an asymmetric shape for the yeast glycogen molecule compared with that of rabbit-liver glycogen. An axial ratio indicating this asymmetry has been calculated.

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REFERENCES

Balls, A. K., Thompson, R. R. & Walden, M. K. (1946). *J. biol. Chem.* **163**, 571.
Balls, A. K., Walden, M. K. & Thompson, R. R. (1948). *J. biol. Chem.* **173**, 9.
Bell, D. J. (1944). *J. chem. Soc.* p. 473.
Bell, D. J., Gutfreund, H., Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **42**, 405.
Bell, D. J. & Manners, D. J. (1951). *Biochem. J.* **49**, lxxvii.
Bell, D. J. & Northcote, D. H. (1950). *J. chem. Soc.* p. 1944.
Bridgeman, W. B. (1942). *J. Amer. chem. Soc.* **64**, 2349.
Daoud, K. M. & Ling, A. R. (1931). *J. Soc. chem. Ind., Lond.*, **50**, 365T.
Errera, L. (1885). *C.R. Acad. Sci., Paris*, **101**, 253.
Halsall, T. G., Hirst, E. L., Hough, L. & Jones, J. K. N. (1949). *J. chem. Soc.* p. 3200.
Halsall, T. G., Hirst, E. L. & Jones, J. K. N. (1947). *J. chem. Soc.* p. 1427.
Hanes, C. S. & Cattle, M. (1938). *Proc. Roy. Soc.* B, **125**, 387.
Harden, A. & Young, W. J. (1912). *J. chem. Soc.* **101**, 1928.
Haworth, W. N. & Percival, E. G. V. (1932). *J. chem. Soc.* p. 2277.
Hirst, E. L., Hough, L. & Jones, J. K. N. (1949). *J. chem. Soc.* p. 928.
Jeanloz, R. (1944). *Helv. chim. acta*, **27**, 1501.
Ling, A. R., Nanji, R. D. & Panton, F. J. (1925). *J. inst. Brew.* **31**, 316.
McAnally, R. A. & Smedley-Maclean, I. (1937). *Biochem. J.* **31**, 72.
Manners, D. J. (1952*a*). Unpublished observations.
Manners, D. J. (1952*b*). *Biochem. J.* **51**, xxx.
Mehl, J. W., Oncley, J. L. & Simha, R. (1940). *Science*, **92**, 132.
Meyer, K. H. & Fuld, M. (1941). *Helv. chim. acta*, **24**, 375.
Northcote, D. H. & Horne, R. (1952). *Biochem. J.* **51**, 232.
Oncley, J. L. (1941). *Ann. N.Y. Acad. Sci.* **41**, 121.
Ostwald, Wo. (1933). *Kolloidzschr.* **63**, 61.
Peat, S., Pirt, S. J. & Whelan, W. J. (1952*a*). *J. chem. Soc.* p. 705.
Peat, S., Pirt, S. J. & Whelan, W. J. (1952*b*). *J. chem. Soc.* p. 714.
Peat, S., Thomas, G. & Whelan, W. J. (1952). *J. chem. Soc.* p. 722.
Record, B. R. (1948). *J. chem. Soc.* p. 1567.
Salkowski, E. (1894). *Ber. dtsch. chem. Ges.* **27**, 3325.
Seifter, S., Dayton, S., Novic, B. & Muntwyler, E. (1950). *Arch. Biochem.* **25**, 191.
Shaffer, P. A. & Somogyi, M. (1933). *J. biol. Chem.* **100**, 695.
Simha, R. (1940). *J. phys. Chem.* **44**, 25.
Stockhausen, F. & Silbereisen, K. (1936). *Biochem. Z.* **287**, 276.
Thorsell, W. & Myrbäck, K. (1951). *Ark. Kemi*, **3**, 323.
Trevelyan, W. E. & Harrison, J. S. (1952). *Biochem. J.* **50**, 298.
Tristram, G. R. (1946). *Biochem. J.* **40**, 721.
Tsuda, S. (1928). *Kolloidzschr.* **45**, 325.
Wilson, R. E. (1921). *J. industr. Engng Chem.* (Ind. ed.), **13**, 326.