Studies on the Polysaccharides of Capsulated Yeasts

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The polysaccharides of the cell wall of brewers' yeasts were investigated by Salkowski (1894) long before Avery, Goebel, Heidelberger and others began their well-known studies on the soluble substances of pneumococci. But while the study of bacterial polysaccharides was subsequently greatly expanded, little progress was made in research on yeast polysaccharides until Zechmeister & Töth (1934) published a paper on the chemical structure of a polyose of the yeast cell wall (yeast 'cellulose'), later studied by Hassid, Joslyn & McCready (1941). Haworth, Hirst & Isherwood (1937) described the structure of a second yeast polysaccharide built up from units of mannose. Preliminary data on the carbohydrates of yeast species other than *Saccharomyces* were published by Kesten, Cook, Mott & Jobling (1930), and Tomcsik (1930) reported on the serological properties of 'gums' derived from a number of yeast species.

As far as we know no data have been published on the polysaccharides of capsulated yeasts. These are distinguished by the formation around the cell wall of voluminous transparent envelopes which remain unstained with ordinary staining techniques, but can easily be demonstrated in Indian ink preparations. This group of yeasts is classified with the genus *Torulopsis* (Lodder, 1938) and includes the pathogenic *T. neoformans* (syn. *Torula histolytica*, *Cryptococcus hominis*, etc.).

In a preliminary note (Aschner, Mager & Leibowitz, 1945), the phenomenon of the elaboration by capsulated yeasts of extracellular starch associated with a pentose-containing fraction was briefly recorded. Biological aspects of this phenomenon are discussed in another paper (Mager & Aschner, 1947); details of the chemical nature of these substances are considered in the present report.

EXPERIMENTAL

Isolation of the yeast starch

Material staining blue with iodine was isolated from a saprophytic yeast identified as *Torulopsis rotundata*. For the preparation of this material cultures were grown under continuous aeration in a medium of the following composition: $(\text{NH}_4)_2\text{SO}_4$, 0.1%; glucose, 1%; KH_2PO_4 , 0.1%; MgSO_4 , 0.05%; aneurin, 20 $\mu\text{g.}/100\text{ ml.}$ After incubation for 4-5 days at 30° the cultures were killed by heating at 60-80° for 0.5 hr. and the cells removed by centrifugation.

The supernatant liquid was concentrated over a steam bath to about one fourth of its original volume and, after cooling, trichloroacetic acid was added to a final concentration of 4 g./100 ml. The slight precipitate which usually appeared after standing overnight in the refrigerator was centrifuged off and discarded, and 3 vol. of ethanol were added to the supernatant. A copious threadlike precipitate was obtained, which was repeatedly washed with 60-70% ethanol acidified by a few drops of acetic acid. When washing fluid was free from reducing sugar, the material was washed three times with absolute ethanol, then with warm ether, and was finally dried in a desiccator. About 1.5 g. of material (1.5% of the original sugar content of the medium) was recovered from a volume of 10 l. of medium.

General properties of the substance

The isolated material showed the characteristic properties of a polysaccharide, viz. it did not pass through cellophan membranes, gave a positive Molisch reaction and failed to reduce alkaline copper solutions except after hydrolysis with dilute acid. The solubility of the substance in water was very slight, but highly viscous and opaque solutions could be obtained by heating for 0.5 hr. under water in an autoclave at 120° at pH 8. The dissolved material was retained by bacterial filters (Berkefeld N and Seitz E.K.). It could be precipitated from aqueous solution by ethanol, acetone or Fehling's solution. The material was free from protein and amino-acids, as determined by the ninhydrin and biuret reactions, and from amino hexoses as determined in its hydrolysate by the reagent of Elson & Morgan (1933). Samples purified by dialysis were N free when tested by the Lassaigue and the micro-Kjeldahl tests, and were ash free on combustion. The blue colour formed with a solution of iodine in potassium iodide disappeared on heating and reappeared on cooling. The quality of the colour was similar to that of the amylose-iodine complex and free from the violet tint characteristic of unfractionated starch containing amylopectin. The light extinction of the iodine complex, measured in a Pulfrich Stufenphotometer, showed a maximum with filter of 610-630 $\text{m}\mu$. wave-length in agreement with the value of extinction for amylose-iodine as reported by Kerr & Trubell (1943) on the basis of spectrophotometric determination. According to the same authors the iodine complex of amylopectin presents a maximum extinction at 700 $\text{m}\mu$.

Estimation of reducing sugar content. Ash-free material (51.6 mg.) was hydrolyzed in 10 ml. of N -HCl for 3 hr. After neutralization with Na_2CO_3 , the solution was made up with water to 20 ml. and the reducing sugar content determined by the method of Somogyi (1945). One ml. of solution containing 2.58 mg. of test material had a reducing value equivalent to 1.69 mg. glucose, i.e. 65.5% of the weight of the substance. The same result was obtained when HCl was replaced by H_2SO_4 or when the strength of the acid was raised (5*N*). Essentially the same result was obtained when the heating time was prolonged to 5 hr.

Chemical nature of the sugar component

Fifteen ml. of a solution of the hydrolyzed material, containing 0.32 mg. of reducing sugar (calc. as glucose)/ml., were fermented with washed commercial bakers' yeast. At the same time a control experiment was done with pure glucose. After 0.5 hr. the solutions were freed from cells by centrifugation and clarified by $Zn(OH)_2$ according to the method of Somogyi (1945). The reducing sugar content was determined, with the following results: control test, 0; test of the unknown, 0.17 mg. Thus, 46.3% of the reducing sugar in the preparation was fermented by bakers' yeast.

To ascertain whether the residual reduction was due to galactose, further fermentation tests were carried out using strains from laboratory cultures of *Torulopsis utilis*, which ferments glucose, fructose and mannose but not galactose; and a strain of *Torulopsis* sp., which ferments galactose as well as the above-mentioned monosaccharides. In a control experiment a mixture containing hydrolyzed amylose, prepared by the method of Ling & Nanji (1923), and galactose was used. The results showed that the galactose-fermenting and the galactose-non-fermenting yeasts each removed about the same amount of sugar. In one experiment the fermentable sugar fraction constituted about 45% of the total reducing sugar. However, the proportion of the fermentable fraction in different preparations varied from 25 to 50% of the reducing value. It may, therefore, be concluded that the blue-staining carbohydrate consists of at least two fractions which can appear in varying proportions in different preparations. One fraction, composed of fermentable sugar, will be designated 'A' and a second, composed of unfermentable sugar, will be designated 'B'.

Action of amylases on the blue-staining material

Suitably buffered 0.05% solutions of the yeast carbohydrate were treated with the following amylase preparations: salivary amylase prepared by the method of Hanes & Cattle (1938); aqueous extract of ground malt, and commercial preparations of taka-diastase and bacterial amylase manufactured by Wallerstein Laboratories. All these amylases caused the blue staining of the material with iodine to disappear. The achromic point was reached much more

slowly with yeast polysaccharide than with soluble or rice starch.

In a quantitative experiment, 10 ml. of a strong solution of yeast starch, containing the equivalent of 5.25 mg. reducing sugar/ml. (calc. as glucose), was treated with 3 mg. of a salivary amylase preparation in the presence of 0.01*N*-acetate buffer pH 6.1 and 3 mg. of NaCl. The mixture was incubated at 37° with constant agitation. After 6 hr. the amount of reducing sugar set free by the action of amylase was found to be 1.04 mg./ml., i.e. 19.8% of the total reducing value. The actual amount of starch present in the material could not, however, be evaluated since the amount of amylase-resistant dextrin was not known.

Decomposition of yeast starch by β -amylase. β -Amylase free from reducing sugar was prepared from wheat by the method of Hanes & Cattle (1938). Yeast carbohydrate (115 mg. containing 69% reducing sugar and 26.3% fermentable sugar) was dissolved in 45 ml. of water by autoclaving at 120° for 30 min. To the viscous solution were added 3 ml. of 0.1*N*-acetate buffer pH 4.8, and 5 mg. of β -amylase. After incubation for 24 hr. at 30° the solution still gave a blue colour with iodine. Reducing sugar was determined in 5 ml. portions before and after fermentation for 2 hr. with bakers' yeast. The results showed 1.46 mg. reducing sugar (as maltose) before, and 0.12 mg. after, fermentation. It may be assumed that the non-fermentable reducing product of β -amylase hydrolysis is dextrin. Deducting this value from the total of reducing sugar released by β -amylase gives 1.34 mg. maltose equivalent to 1.41 mg. glucose. The amount of fermentable sugar in this preparation equalled 26.3% of the weight of the substance. Hence, on the assumption that the fermentable sugar is wholly derived from the starch fraction, complete hydrolysis by β -amylase should have set free from 11.5 mg. of the substance an amount of maltose equivalent to 3.02 mg. glucose. The amount actually obtained (1.41 mg.) represents, therefore, 46.7% of the starch fraction. (Since it was subsequently found that the capsular material which appears in association with the yeast starch contains about 15% of zymohexose, the real value for maltose split off by β -amylase would be somewhat higher.)

It has recently been claimed (Wilson, Schoch & Hudson, 1943) that the presence of fatty materials in corn starch prevents the complete conversion of its amylose component by β -amylase or *B. macerans* amylase. After the removal of lipids with 80% (v/v) methanol, complete enzymic hydrolysis of corn starch-amylose can be obtained. We, therefore, repeated the experiments described above using a preparation of yeast starch which had previously been treated with 80% (v/v) methanol. The results were in no way altered.

Examination of the product of amylase action. It remained to be determined whether or not the fission product obtained by amylase action was maltose. The colorimetric method described by Hestrin & Mager (1946, 1947) for the identification of sugars was used for this purpose.

Absolute ethanol (3 vol.) was added to a solution of yeast starch treated with salivary amylase as described above. The resulting precipitate was separated by centrifugation and the supernatant fluid evaporated to dryness. The residuum was redissolved in 5 ml. of distilled water. This solution was found to contain 4.3 mg./ml. of reducing sugar calculated as maltose. The solution (0.6 ml.) was transferred to a Thunberg tube and evaporated to dryness. To the dry residue were added 4 ml. of phosphoric acid (85%), and the solution heated in a boiling water bath for 30 min. No colour formation occurred. Finally, about 10 mg. of pyrocatechol was added to the phosphoric acid solution and the heating continued for 10 min. A red-violet colour then appeared and proved to be identical in absorption curve with that of glucose and glucose anhydrides.

Since the product of amylase hydrolysis gave a negative reaction with the monose reagent of Tauber & Kleiner (1932), it was obvious that the sugar in question must be an oligosaccharide. Direct support for its identification as maltose was obtained by the preparation of a phenylosazone, which was soluble in hot water and yielded yellow crystals of the microscopic form described by Hassid & McCready (1942) for maltosazone.

Search for zymohexoses other than glucose

The experiments so far reported do not exclude the possibility that the 'A' fraction contains fructose or mannose in addition to glucose. Quantitative tests for fructose (Seliwanoff, Pinoff) were negative. Aldose determination of the hydrolyzed material by the iodometric method of MacLeod & Robison (1929) gave a result closely approximating the value for total reducing sugar obtained by Somogyi's method.

As a test for mannose, the isolation of mannose phenylhydrazone, a substance distinguished by its insolubility and the rapidity of its formation in the cold, was attempted. Although about 200 mg. of hydrolyzed material were used for this test, no precipitate other than a small amount of a non-crystalline red sediment was produced with phenylhydrazine. The result of this test was, therefore, read as negative.

Identification of fraction 'B'

The hydrolyzed material freed from zymohexose (fraction 'B') was subjected to various qualitative tests. As little as 1–2 mg. of material gave a strong positive reaction with Bial's orcinol reagent, which gives positive tests with uronic acids and methylpentoses as well as with pentoses; but the presence of compounds of the first two groups was excluded by

specific tests. The naphthoresorcinol test of Tollens was applied for uronic acid and the acetone test of Rosenthaler for methylpentose (cf. Van der Haar, 1920; Browne & Zerban, 1941). Both tests were negative even when 50–100 mg. of hydrolyzed material was employed. On the other hand, the aniline acetate test of Schiff, which is fairly specific for pentose, was strongly positive with 1–2 mg. of the hydrolyzed polysaccharide. In every case control tests on solutions of known sugars including xylose, glucuronic acid and rhamnose were run.

The results indicated clearly that the Bial positive sugar in fraction 'B' is a pentose. The question arose whether all the unfermentable sugar fraction consists of pentose.

The material (21 mg.) was hydrolyzed with hot N-HCl for 2.5 hr. After neutralization the solution was adjusted to 50 ml. The reducing sugar content was then determined on 2 ml. of this solution. In another 2 ml. diluted 1:80 the pentose content was estimated by the method of Mejbaum (1939). The quantitative method of Mejbaum for pentose was chosen for its simplicity and because it affords results which are independent of the configuration of the pentose (Schlenk, 1943). The photometric readings were made in a Lumetron apparatus using filter 610 m μ .

The following results, calculated on the volume after hydrolysis and adjustment to 50 ml., were obtained: total reducing sugar: 0.215 mg./ml.; pentose: 0.206 mg./ml. The values for pentose and total sugar were thus practically identical, the slight difference observed being within the limits of experimental error. The conclusion seems warranted that all of the unfermentable sugar in fraction 'B' is pentose.

Determination of the specific optical rotation of the hydrolyzed material

The optical rotation was determined in a 1 dm. tube at 18° after the addition of alkali to a concentration of 0.1N. The results are summarized in Table 1.

Table 1. *The specific rotation of the unfractionated hydrolysate of carobohydrate material and fraction 'B'*

	Sugar conc. (c) (%)	Optical rotation (α)	$[\alpha]_D = 100\alpha/lc$
Unfractionated material	1.28	+0.60°	+46.8°
Fraction 'B'	0.67	+0.13°	+19.4°

The rotation values of different dextrorotatory pentoses, as reported in the literature, are as follows: D(+)-xylose +10°; D(+)-lyxose +13.9°; L(+)-arabinose +105.5°; L(+)-ribose +23.7°. The rotation value of fraction 'B' is, therefore, nearest to D-xylose and L-ribose. However, the presence of a certain amount of another pentose, in particular of

Table 2. *Fermentation of various pentoses and of the hydrolysate of fraction 'B' by bacterial species of the family Enterobacteriaceae*

Species of bacteria	L-Arabinose	D-Arabinose	D-Xylose	D-Ribose	D-Lyxose	Pentose of the capsule material
<i>Salmonella choleraesuis</i>	-	-	A, G	A, G	±	A, G
<i>S. schottmülleri</i>	A, G	-	A, G	A, G	±	A, G
<i>S. typhimurium</i>	A, G	-	A, G	A, G	-	A, G
<i>S. paratyphi</i> (A)	A, G	-	-	A	±	-
<i>S. pullorum</i>	A, G	-	-	A, G	-	-
<i>Shigella ambigua</i>	-	-	-	A	±	-
<i>Shig. sonnei</i>	A	-	-	-	±	-
<i>Proteus Oxy</i>	-	-	-	-	-	-
<i>Escherichia coli</i>	A, G	A, G	A, G	A, G	±	A, G
<i>Eberthella typhosa</i> str. Bhatnagar	-	-	A	A	A	A

Explanation of signs: A = acid; G = gas; ± = weak acidification; - = no acid nor gas.

lyxose, cannot be excluded. (Gulland & Barker (1943) found a small amount of L-lyxose in commercial preparations of yeast nucleic acid.) The higher rotation of the unfractionated material, on the other hand, is consistent with the presence of glucose in high concentration.

Microbiological identification of the pentose component

With only a limited amount of material at our disposal detailed investigation of the configuration of the pentose by the accepted preparative methods was not feasible. We attempted to avoid this difficulty by means of a microbiological method which could be applied to small quantities. Although many microbiological methods have been suggested by various workers for the identification of different sugars (cf. Harding & Nicholson, 1933) no complete scheme for the identification of pentose configurations has been elaborated.

It is known that various species in the family of Enterobacteriaceae differ from one another in their ability to ferment D-xylose and L-arabinose. However, information concerning the behaviour of bacteria of this group towards the other configurational isomers of pentose has not, to our knowledge, been published. For this reason we examined the behaviour of a number of species towards a collection of pentoses.

The tests were made in test tubes (small size) containing 3 ml. of semi-solid agar of the following composition: peptone, 0.2%; Na₂HPO₄, 0.07%; NaCl, 0.1%; Andrade indicator; and 0.1-0.2% of the sugar to be examined. It was found that the given low concentration of sugar sufficed for acid and gas formation in positive cases.

The ability of the bacteria to ferment the pentose contained in fraction 'B' of the carbohydrate material of *T. rotundata* was determined as follows: The hydrolyzed material was adjusted to pH 7.0 and freed from zymohexoses by yeast fermentation. The cleared solution was diluted to a sugar concentration of 0.4% (calculated as glucose). The solution was sterilized by passing through a bacterial filter and was added to an equal volume of double strength semi-solid agar.

The results of these examinations, summarized in Table 2, show that the fermentation behaviour of the pentose component constituting fraction 'B' was identical with that of D-xylose.

Identification of fraction 'B' with the capsular substance of Torulopsis rotundata

It was mentioned in a preliminary note (Aschner, Mager & Leibowitz, 1945) that under certain conditions a pentose-containing polysaccharide which does not stain blue with iodine can be isolated from cultures of *T. rotundata*. The production of the iodine-staining material is suppressed when a source of nitrogen is employed which does not allow acidification of the culture medium such as, for instance, 0.05% urea, 0.1% KNO₃ or 0.1% asparagine. The substance produced under these conditions could be shown to be derived from the capsule of the yeast, since it appeared in cultures of all the non-fermenting capsulated yeasts, but failed to appear in cultures of their capsule-free variants (Mager & Aschner, 1947).

Thus it occurred to us that fraction 'B' might be identical with the capsular substance. The experimental data summarized in Table 3 seem fully to confirm this assumption.

Table 3. *Composition and properties of the capsular substance*

- (i) Hot Fehling's solution: precipitate; no reaction
- (ii) Total reducing sugar of the hydrolysate: 58-67% of the weight of the substance
- (iii) Fermentable fraction of the total reducing sugar: 13.5-15%
- (iv) $[\alpha]_D$ of the unfermentable 'B' fraction: +20.6 ± 2.2°
- (v) Action of amylase: not acted on
- (vi) Qualitative tests on the unfermentable fraction: pentose
- (vii) Meijbaum's pentose estimation: 61% of the weight of the substance
- (viii) Microbiological test for pentose: D-xylose
- (ix) Tests for mannose, fructose and galactose: negative

One additional fact came to light in these experiments, viz. that the capsular substance itself con-

tains a fraction composed of a zymohexose obviously identical with glucose. This fraction however must be different from starch, since it does not impart a blue colour to the material when treated with iodine, and since it is not attacked by amylases.

Elementary analysis of the capsular substance. In various preparations the amount of the reducing sugar yielded by hydrolysis constituted 60–70% (w/w) of the substance. This seems to indicate that the material isolated from *T. rotundata* contains an additional non-carbohydrate fraction. Since, however, it might be that the apparent deficit in reducing sugar was due to incomplete hydrolysis or to some other technical factor, the chemical nature of the material as a whole was examined by an elementary analysis.

Duplicate microanalyses carried out on a sample of capsular substance yielded the following results:

Material dried at 96°: C, 38.1, 38.5; H, 6.7, 6.7%; N, nil; ash, nil.

Material dried at 108°: C, 38.9, 38.8; H, 7.2, 7.4%.

The theoretical values for hexosan and pentosan are:

Hexosan, C, 44.4; H, 6.17%; *pentosan,* C, 45.45; H, 6.0%.

There is thus considerable divergence between the experimental findings and these theoretical values.

Isolation of the yeast starch free from capsular fraction 'B'

Several attempts to prepare a yeast starch preparation free from accompanying capsular substance were unsuccessful. As mentioned above, hot Fehling's solution precipitated the capsular substance. We therefore attempted to isolate starch from the supernatant fluid after treatment with Fehling's solution. It was found, however, that the bulk of the starch fraction was trapped by the precipitate of the capsular substance.

We succeeded in separating the yeast starch from the capsular material by a freezing-out procedure. Culture medium in which *T. rotundata* has been grown was freed from cells and concentrated to about one tenth of its original volume. After freezing for 48 hr. the starch constituent settled out whereas the capsular substance remained in solution. In this way about 80 mg. of pure yeast starch were recovered from 3 l. of culture medium. Solutions of the pure material were distinguished by rapid retrogradation on standing. In contrast to the mixed preparation the pure yeast starch was precipitated by an excess of iodine solution and not by Fehling's solution. On hydrolysis 22 mg. of the material yielded 20.5 mg. of reducing sugar (c. 90% of theory). The hydrolyzed sugar was completely fermented by bakers' yeast.

Examination of the carbohydrate materials produced by other species of capsulated yeasts

Since the ability to produce an extracellular material staining blue with iodine is shared by non-fermenting capsulated yeasts as a group (Mager & Aschner, 1947) the experiments described were repeated on a number of other capsulated yeasts. The results were not essentially different from the findings with *T. rotundata* reported above. The blue-staining material and the capsular substance elaborated by the pathogenic yeast *T. neoformans* (Palestine strain) was essentially identical with the analogous substances from *T. rotundata*. In examining the carbohydrate materials isolated from other capsulated non-fermenting species we confined ourselves to tests of their ability to stain blue with iodine, of their susceptibility to hydrolysis by salivary amylase and of their association with a material containing pentose. In all these respects the results with the following species of *Torulopsis*, comprising all those tested, were the same as with *T. rotundata*: *T. innocua*; *T. mucorugosa*; *T. liquefaciens*; *T. neoformans*, str. Sanfelice; *T. neoformans*, str. Tartar; *T. neoformans*, str. Freeman & Weidman.

Demonstration of a starch-synthesizing enzyme in cells of Torulopsis rotundata

As reported by Mager & Aschner (1947), resting cells of *T. rotundata* are incapable of producing starch. From this it was inferred that the elaboration of starch is in some way linked with vital anabolic processes of the proliferating cells. Nevertheless, it appeared probable that the enzymic apparatus of starch formation might be set free by destruction of the cellular organization. Attempts to release the enzyme were first made by autolyzing the cells, but the cells of *T. rotundata* proved to be extremely stable in this respect, since cells kept under toluene at 37° showed signs of cell disintegration only after a period of at least 2 weeks.

The following experiment was then undertaken: cells of *T. rotundata* grown at 30° for 3 days on peptone glucose agar, i.e. under conditions which do not permit the production of starch, were harvested with aseptic precautions and washed free of the culture medium. Glucose or glucose-1-phosphate (0.5%) was added to thick suspensions of these cells in 5 ml. acetate buffer solutions of graded pH, and the mixtures incubated under toluene. Samples (0.2 ml.) were withdrawn and tested for starch with iodine solution. After 20 days of incubation, tests showed that starch was produced with Cori ester as substrate over the range pH 4.5–5.9, but not with glucose. In both instances marked autolysis was evident microscopically. It seems obvious, therefore, that the disintegrating cells release a starch-producing enzyme which acts as a phosphorylase.

This enzyme could also be demonstrated in cell preparations of *T. rotundata* by another method. Cells grown on glucose peptone agar were dried from the frozen state in a Cryochem apparatus. About 0.5 g. of the dried material, washed twice with acetone and ether, was mixed with an equal amount of glass powder and ground for several minutes in a glass mortar with 5 ml. of water. After centrifugation the supernatant was added to an equal amount of 1% of either glucose or Cori-ester solution in acetate buffers of varying pH. After 5–10 min. the solutions containing Cori-ester at pH 4.5–5.9 turned blue when tested with iodine.

DISCUSSION

The chemical analysis of materials which stain blue when treated with iodine and which were isolated from liquid cultures of the yeast *T. rotundata* and related capsulated species, shows that these materials are mixtures of at least two different carbohydrate substances. One is a polyglucoside similar to plant starch, and the other is a substance derived from the yeast capsule. The physical and biochemical properties of the polyglucoside as examined in the presence of the capsular substance are not identical with the characteristics of either of the two fractions of plant starch. In its reaction to iodine, its low solubility and its tendency to retrogradation in neutral solutions, yeast starch resembles amylose (β -amylose, amyloamylose). On the other hand, the incompleteness of its conversion by β -amylase suggests that it should be classified with amylopectin (α -amylose, erythroamylose; Samec & Waldschmidt-Leitz, 1931; Hanes, 1937; Hassid & McCready, 1943). It seems likely that yeast starch is a substance intermediate between α -amylose and β -amylose, and possibly identical with the γ -amylose component of corn starch described by Kerr & Severson (1943). However, the possibility cannot be ruled out that the properties of yeast starch in the examined preparations were dependent on the close association of the starch fraction with capsular substance. It is conceivable that amylase cleavage is hindered by the presence of the capsular material.

Cori, Schmidt & Cori (1939) have shown that phosphorylase isolated from skeletal muscles produces pure amylose, whereas live muscle produces glycogen. To explain this difference Cori & Cori (1944) (cf. Cori, Swanson & Cori, 1945) put forward the theory that glycogen is produced by the simultaneous action of two enzyme systems, the first forming the straight chain of the amylose molecule by binding glucose units in 1:4 bonds, and the second attaching to this chain the branches characteristic of glycogen which are linked by 1:6 bonds. In the course of making enzyme preparations, the 'branching factor' may be lost with the result that only the

'straight chain factor' remains in the enzyme preparation finally obtained. A similar view was suggested by Haworth, Peat & Bourn (1944; cf. Bourn & Peat, 1945) to explain the absence of the amylopectin-forming factor in phosphorylase prepared from potatoes by the method of Hanes (1940).

Speculating along such lines we might assume that the 'branching factor' in the case of the yeast cells is destroyed or inactivated at the low pH at which starch production occurs, while the 'amylose-producing factor' retains its activity in the same medium. Since the cell wall is injured by the acid reaction of the medium, the amylose is able to pass out of the cell and accumulates in the capsule and in the medium. The assumption that the cell wall suffers damage under conditions of progressive acidification is supported by microscopic observations (Mager & Aschner, 1947).

The hypothesis considered above does not explain the fact that starch production is limited to a special group of yeasts. Another interpretation which offers a more satisfactory explanation for the whole phenomenon deserves consideration. According to this hypothesis it is assumed that starch is the precursor of the yeast capsule pentosan. Under normal growth conditions the starch may be transformed into pentosan by oxidation of the glucose units at carbon 6 to glucuronic acid, with subsequent decarboxylation of the glucuronic acid to D-xylose. This transformation may be supposed to be paralyzed at an acid pH, resulting in the fixation of the intermediary starch. It will be recalled that a mechanism entailing mild oxidation and decarboxylation has been envisaged by various authors to explain the mode of formation of the pentose constituent of hemicellulose and pectins in plants (cf. Norman, 1929). Such an interpretation would explain two facts which are not well accounted for in the alternative hypothesis: the selective accumulation of the starch in the capsule, and the restriction of the phenomenon to capsulated yeasts.

SUMMARY

1. The properties of a carbohydrate material which stains blue with iodine, isolated from cultures of *Torulopsis rotundata*, *T. neoformans* and other capsulated yeasts, are described.

2. The material is a mixture of starch and pentosan derived from the capsule of the yeast.

3. The capsular material consists of a large carbohydrate fraction (c. 60–70%) and a non-carbohydrate one. The carbohydrate constituent is composed of a zymohexose (15%) and a pentose (85%). The reported experiments have led to a tentative identification of the zymohexose as glucose and of the pentose as D-xylose.

4. The demonstration in cells of *T. rotundata* of a phosphorylase which synthesizes starch is recorded.

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Determination of Pregnanediol in Urine for Diagnostic Purposes

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Pregnanediol, excreted in urine in the form of sodium pregnanediol glucuronidate, is the chief metabolite of the corpus luteum hormone, progesterone, and the relation between pregnanediol excretion and corpus luteum activity makes it important to have a reliable and convenient method for determination of pregnanediol. It is physiologically inactive, in spite of its close relation to the hormone, and biological tests cannot, therefore, be used. Pregnancy may be diagnosed at an early stage from the pregnanediol excretion, which is, during the first 2 months' gestation, about equal to the maximum in the latter half of the menstrual cycle, rises progressively to a maximum and then falls precipitously

after delivery. Pregnanediol excretion drops to a low level when miscarriage is imminent (cf. Guterman, 1946). Also, the level of pregnanediol excretion may be considerably increased in adrenal cortical carcinoma or adrenal hyperplasia (Venning, Weil & Browne, 1939).

Various methods for the determination of pregnanediol in urine have been proposed, but they are either too complicated or too inaccurate for routine clinical use. The method of Venning & Browne (Venning, 1937, 1938; Venning & Browne, 1940) and the modification of Westphal (1944) do not, in the author's experience, allow the determination of less than 5 mg. of sodium pregnanediol glucuronidate,