Studies on Yeast Metabolism

7. YEAST CARBOHYDRATE FRACTIONS. SEPARATION FROM NUCLEIC ACID, ANALYSIS, AND BEHAVIOUR DURING ANAEROBIC FERMENTATION*

BY W. E. TREVELYAN AND J. S. HARRISON

Research and Development Department, Distillers Co. Ltd., Epsom, Surrey

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Yeast contains trehalose (Myrbäck, 1949) and at least three polysaccharides (Whistler & Smart, 1953): glycogen, mannan and an insoluble glucan. Trehalose and (in part) glycogen function primarily as an energy reserve, whereas mannan and glucan are structural components located in the yeast-cell wall (Northcote & Horne, 1952; Kreger, 1954). Chitin is another constituent of the cell wall, but as hexosamines do not react with the anthrone reagent (Chung & Nickerson, 1954) its estimation is not considered here. The polysaccharides are usually isolated by methods described originally by Salkowski (1894), though some workers are critical of the drastic chemical treatments involved (Sevag, Cattaneo & Maiweg, 1935).

Trevelyan & Harrison (1952) applied Dreywood's anthrone reagent (Dreywood, 1946) to the determination of yeast carbohydrates. The four yeast carbohydrates mentioned were assumed to be the sole source of anthrone-positive material. On the evidence provided by paper chromatography, and later corroborated by ion-exchange chromatography of borate complexes (Forrest, Trevelyan & Harrison, unpublished work), trehalose was estimated as carbohydrate extracted by cold trichloroacetic acid. Mannan was separated as an insoluble copper complex hydrolysable to mannose. Carbohydrate remaining in the cell debris after extraction with H_2SO_4 until the cells failed to react with a periodic acid-Schiff stain was taken to be glucan. All other polysaccharide, which gave glucose after hydrolysis, was reported as glycogen. This material actually appeared in two separate fractions. Its partition into alkali- and acid-soluble forms, though reproducible, is, however, of doubtful significance, in view of the chemical identity of glycogen extracted from yeast in different ways (Northcote, 1953).

Trevelyan, Forrest & Harrison (1952) subsequently simplified this procedure, and similar methods have recently been described by Chung & Nickerson (1954) and by Yemm & Folkes (1954). One drawback of the method, however, remained: the sum of carbohydrate recovered in the separate

fractions was always slightly less than the 'total carbohydrate' figure obtained by applying the anthrone technique to whole yeast. In an empirical procedure this is not a serious fault, but an internal check against large accidental error is undoubtedly useful. Again, in some steps the yeast cells required longer and more careful centrifuging than was desirable in routine work.

Revision of the carbohydrate fractionation procedure to meet these criticisms had, as a further aim, its extension to include the separation and determination of nucleic acid. This seemed feasible, since standard methods for nucleic acid analysis (Volkin & Cohn, 1954) are based on extraction by acid and alkali, as was the carbohydrate fractionation method. Yeast contains relatively little deoxyribonucleic acid (Bourdet, Mandel & Guillemet, 1951), and all material reacting as pentose which can be extracted from yeast after removal of adenosine 5'-phosphates and other coenzymes with cold trichloroacetic acid may therefore be regarded without great error as ribonucleic acid.

METHODS

Preparation of yeast for analysis

Samples (5 g.) were taken as required from a master sample of commercial pressed baker's yeast stored in the refrigerator. For the evaluation of fractionation procedures the yeast was suspended in water, a convenient amount of the suspension (containing 100 or 200 mg. of yeast) was centrifuged and the supernatant discarded.

Determination of carbohydrate with anthrone

The anthrone reagent contained 2 g. of anthrone dissolved in a mixture of 75 ml. of water and 950 ml. of H_2SO_4 . The composition of the reagent selected was that which had been found to give maximum colour development with glucose (see Results) since three of the four yeast carbohydrates are glucose polymers. Carbohydrate solutions were delivered into up to twenty-four Pyrex tubes (2.5 cm. $\times$ 15 cm., 28 g. wt.) and water was added to a final volume of 3.8 ml. Anthrone reagent (8.2 ml.) was then run down the side of the tube from a 50 ml. burette; because of the viscosity of the reagent the burette outlet had to be fairly large. The tube was then held by the lip with the hand and rotated quickly by wrist action for 7 sec. The tube was

* Part 6: Trevelyan & Harrison (1956b).

then replaced in the rack, a second tube was taken, and so on. With a little practice it was found possible to handle tubes at exactly 0.5 min. intervals, so that the time needed to be noted only at the beginning of each batch of six, when the burette had been refilled. After 10 min. each tube was transferred in turn to a rack standing in running tap water and left to cool for 5 min. Each batch of tubes included duplicate glucose (0.5 μ mole) and mannose (1 μ mole) standards. Optical density at 620 m μ . was measured with the Unicam DG. 350 spectrophotometer, set to zero against a reagent blank. In recent work it has been found that the optical density of concentrated sulphuric acid solutions, though not of aqueous solutions, alters appreciably with a change in the radial positioning of the 0.75 in. tubes used with this instrument. Reproducibility is better if the spectrophotometer is set to zero against distilled water, and the optical density of solutions (including the reagent blank) are measured with a tube on which the radial position giving maximum reading is indicated by a scratch.

Pentose determination with orcinol

Both intensity and quality of colour deteriorated when sulphuric acid was substituted for hydrochloric acid in orcinol reagents containing either ferric (Mejbaum, 1939) or cupric salts (Barrenscheen & Peham, 1941). Attempts to develop a heat-of-mixing technique for pentose determination similar to that used with anthrone were in consequence unsatisfactory. To determine ribonucleic acid pentose (RNA pentose) an adaptation of the Mejbaum procedure was used.

The test solution (5 ml., containing up to 1.5 μ moles of RNA pentose) was delivered into a tube graduated at 12.5 ml. ('E-MIL' tubes for blood-sugar determination) followed by 0.2 ml. of 10% (w/v) ethanolic orcinol (Albaum & Umbreit, 1947). The latter solution was preferred to orcinol in HCl when, as in the present investigation, only a small number of determinations were made at one time. Orcinol solution was followed by 5 ml. of HCl reagent (prepared by pipetting 2 ml. of 1% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ into a dry 200 ml. volumetric flask, and making up to volume with conc. HCl), making a total volume of 10.2 ml. After mixing, the tubes were covered with glass stoppers, heated for 20 min. at 100° in a water bath, and cooled in running water after removing the stoppers. If excess of hexose had resulted in turbidity, this was cleared by making up to 12.5 ml. with ethanol, before measuring the optical density at 660 m μ ., otherwise no adjustment of volume was made. The standard was a freshly made solution of yeast RNA (25 mg. + 2 ml. of N-NaOH in 100 ml.), which itself was standardized by phosphorus determination (method of Gomori, 1942) as recommended by Schneider (1945).

Fractionation scheme

Total carbohydrate. Washed yeast (100 mg., see above) was transferred to a 200 ml. volumetric flask containing 4 ml. of 0.5M trichloroacetic acid (TCA) and kept for analysis. Just before estimation the flask was shaken to suspend the yeast, and water added to 200 ml. After mixing by inversion, about one-third of the suspension was discarded, and the remainder vigorously shaken to ensure homogeneity before withdrawing about 1 mg. of yeast for anthrone estimation.

Total yeast carbohydrate measured in this way includes

the hexose polymers glycogen, glucan, mannan and trehalose. Ribonucleic acid, the chief pentose-containing constituent of yeast, reacts with anthrone to give a transient green colour, which fades rapidly under the conditions of the estimation. The residual optical density at 620 m μ . corresponds to an inaccuracy in the total carbohydrate figure of <1%, as may be seen from the entries in Table 1 under the headings 'total carbohydrate' and 'hexose in RNA fraction'. The colour produced by mixtures of hexoses and pentoses was found to be strictly additive, and no interference of pentose with hexose estimation of the type described by Johanson (1953) was encountered. Thus, if the perchloric acid extraction step (38°) of the fractionation scheme was omitted, RNA appeared in the 'alkali-soluble' fraction together with mannan and some glycogen, with negligible effect on the 'alkali-soluble carbohydrate' as estimated by anthrone.

Trehalose. A duplicate sample of 100 mg. of washed yeast was suspended in 4 ml. of 0.5M TCA and kept for 1 hr. in an ice bath. The suspension was frequently shaken; if the yeast was allowed to settle, trehalose extraction was not complete, as shown by the appearance together with RNA, in the next fraction, of significant amounts of hexose. After centrifuging, the cells were washed once with 4 ml. of water, and the combined supernatants made up to 25 ml. A portion (1 ml.), equivalent to 4 mg. of yeast, was taken for estimation by anthrone. TCA-soluble phosphate was determined, after digestion of 10 ml. of extract with HClO_4 , by the method of Gomori (1942).

Nucleic acid (RNA). When all yeast samples (usually four) had been extracted with cold TCA, the cells were suspended in 4 ml. of 0.5M- HClO_4 and incubated for 90 min. at 38°. Occasional shaking kept the cells in suspension. During this time estimation of total carbohydrate and trehalose was completed, the figures being useful as a guide to the correct dilution (to ensure optical densities close to that of the glucose standard) of glycogen fractions obtained later. After centrifuging and washing the cells with 4 ml. of water the HClO_4 extracts were made to 20 ml., when 3 ml. portions (15 mg. of yeast) were taken for pentose and hexose determinations, and 5 ml. for determination of HClO_4 -soluble phosphate by HClO_4 digestion and phosphate analysis by the Gomori (1942) method.

Mannan + alkali-soluble glycogen. After dispersing the cell residue in 4 ml. of 0.25M- Na_2CO_3 , the tubes were covered with glass stoppers and heated for 45 min. at 100° in the water bath. After brief cooling the cells were centrifuged and washed once with 4 ml. of water. The alkali extract was made up to 10 ml., and 4 ml. was diluted to a convenient volume for determination of mannan + alkali-soluble glycogen.

Mannan. A second 4 ml. of alkali extract was transferred to a centrifuge tube containing 0.4 ml. of 10M-KOH or NaOH, and 2 ml. of Fehling's solution was added. After mixing, the tubes were stoppered and left overnight. After centrifuging down the mannan-copper complex, it was washed with 3 ml. of 0.1M-NaOH, dissolved in 1 ml. of M- H_2SO_4 , and then made up to 25 ml., of which 3 ml., equivalent to 4.8 mg. of yeast, was taken for anthrone analysis.

Acetic acid-soluble glycogen. The cell debris from the alkali-extraction step was suspended in 4 ml. of 2M acetic acid and heated for 2 hr. in a boiling-water bath. After brief cooling, centrifuging and washing, the extract was diluted to a convenient volume.

HClO₄-soluble glycogen. The cell residue was then extracted in a similar fashion for 30 min. at 100° with 0.5M-HClO₄. It was found advisable not to attempt complete dispersion of the cells when washing with water, as otherwise there was a danger of carrying over some yeast when decanting the supernatant.

Glucan. 2M-NaOH (2 ml.) was added, the cells were suspended roughly by shaking and the suspension was transferred to 50 ml. cylinders having a generous air space above the mark. The cylinders were stoppered and shaken violently a few times, when a homogeneous suspension was obtained which did not settle even after standing overnight; 2 ml. (4 mg. of yeast) was taken for analysis.

Determination by anthrone of mannan+alkali-soluble glycogen, acetic acid and HClO₄-soluble glycogen, and glucan, was now completed, and the data were examined to see that the sum of the individual fractions checked with the total carbohydrate figure, and that optical densities were not too high for Beer's law to apply. On the following day any suspect determinations were repeated when mannan estimation was performed. Pentose and phosphorus determinations were also completed.

Expression of results. Mannan was evaluated by comparison with (duplicate) glucose and mannose standards, prepared freshly by diluting stock solutions of the sugars in saturated benzoic acid. Other carbohydrate fractions were estimated by comparison with glucose, though a single mannose standard was included with each batch of anthrone determinations as a check on the constancy of the glucose/mannose colour ratio. Results are reported as μ moles of hexose/g. of yeast. Total yeast carbohydrate expressed as μ moles of hexose/g. of yeast was computed as follows:

$$\begin{aligned} \text{total (as hexose)} &= \text{total (as glucose)} \\ &+ \text{mannan (as mannose)} - \text{mannan (as glucose)}. \end{aligned}$$

Application of the fractionation scheme to four yeast samples took 2 working days.

Shortened fractionation scheme. A study of the changes in the various fractions during fermentation (see Results) suggested that there is no clear distinction between the various forms of glycogen. For the routine analysis of yeast carbohydrates it is therefore suggested that the acetic acid-extraction step be omitted and the sum of alkali- and HClO₄-soluble glycogen be reported as 'glycogen'.

Fermentation technique

In a study of the changes during anaerobic fermentation of yeast carbohydrates, nucleic acid, and TCA- and HClO₄-soluble phosphate, 2.5 g. of yeast in 80 ml. of medium was stirred by a stream of N₂ (150 ml./min.). After 10 min., when the suspension had attained the temperature of the thermostatic bath (30°), fermentation was initiated by adding 20 ml. of glucose (concentration after mixing, 0.3M), mannose (0.3M), or maltose (0.15M). After addition of sugar the full medium contained 0.05M sodium succinate buffer, pH 5.5, 5 mM-MgCl₂, 2 mM-KH₂PO₄, 8 mM-KCl, 0.01M-(NH₄)₂SO₄, 0.02M DL-asparagine and 5 μ M thiamine. N-free medium lacked asparagine or (NH₄)₂SO₄.

In a further study of the effect of some constituents of these media, the yeast was suspended in succinate buffer, and other salts were added as listed in the legend to Fig. 9.

The fractionation scheme was applied to 4 ml. samples withdrawn after 30, 60 and 120 min., and centrifuged, and

washed free of medium by centrifuging after suspension in 5 ml. of water (twice). The composition of the yeast immediately before addition of sugar was also determined. Results are given as μ moles/g. original weight of yeast.

RESULTS

Determination of carbohydrate with anthrone

Temperature during colour development was varied by altering the distribution of water between the sugar solution and the anthrone reagent; the composition after mixing was, however, kept constant. A basic anthrone reagent was prepared containing 0.4 g. of anthrone in a mixture of 10 ml. of water and 190 ml. of H₂SO₄. A series of reagents of graded water content were prepared by adding to 8 ml. of basic reagent contained in each of a number of Pyrex tubes 0, 0.2, ..., 2.0 ml. of water (say x ml.), mixing and cooling. Sugar solution, together with sufficient water to make (4- x) ml. added in all, was layered on top, and the contents were mixed and optical density was determined after 10 min. colour development (see Methods).

As Fig. 1 shows, the composition of the anthrone reagent giving maximal colour development varied with the nature of the sugar. The optimum for ribose shifted to a higher

Fig. 1. Effect of temperature, controlled by adding water to 8 ml. of anthrone reagent, on the colour produced by 1 μ mole of sugar. x, Fructose; ●, glucose; ○, mannose; □, xylose; + (full line), ribose; + (broken line), ribose with low-anthrone reagent.

temperature when the concentration of anthrone was reduced to one-eighth, indicating that destruction of colour at higher temperatures may involve oxidation by anthrone itself.

The composition of the reagent optimum for a particular sugar will, of course, change slightly if alteration is made in conditions affecting colour development, e.g. the volume after mixing, or the type of tube used. However, the results of Fig. 1 can be reproduced in a short time, and the correct composition for any particular set of conditions can be found.

The heat-of-mixing technique will give accurate results only if the procedure adopted for mixing reagent and carbohydrate solution is rigidly standardized. By use of the simple technique described in Methods, and the appropriate reagent, the optical density of 0.5 μ mole of glucose was found reproducible to $\pm 1\%$ (s.d. of ten samples). Over a period of a month the variation was only $\pm 2\%$ (thirty samples). An equivalent amount of mannose developed, with this reagent, 50–55% as much colour, the figure depending on the atmospheric temperature.

By the use of two anthrone reagents of different composition, it was found possible to determine fructose and glucose in a mixture of the two sugars. Mixtures of mannose and glucose, or ribose and glucose, could not be satisfactorily analysed. In general, it was found that results with a given sugar became progressively more variable the more reagent-water content was increased above the optimum. Consequently analysis of mixtures is feasible only when the colour/ μ mole developed by the weaker reagent differs widely for the two sugars.

Examination of procedures for the separation into fractions of yeast carbohydrates and nucleic acid

Extraction of trehalose by TCA or Cetavlon (cetyltrimethylammonium bromide). Yeast was extracted by shaking on a mechanical shaker at room temperature with (a) 4 ml. of 0.5M TCA, or (b) 1 ml. of M TCA and 2 ml. of ethanol, or (c) 3.5 ml. of water containing 10 mg. of the cationic detergent Cetavlon. After a definite time the cells were centrifuged and washed with TCA, ethanolic TCA, or water respectively, and carbohydrate in the supernatants was determined. After 60 min. for method (a), 15 min. for (b), or 15 min. for (c), carbohydrate extracted by the different methods had reached a maximum, which was approximately the same in each case (Fig. 2). (Contrast extraction of phosphate by TCA and cationic detergents; Kamen & Spiegelman, 1948.) This agreement shows that partitioning yeast carbohydrate by cold TCA is not entirely arbitrary, and that no appreciable amount of (ethanol-insoluble) polysaccharide is extracted by aqueous TCA. In general we have used method (a), as the extracted cells can then be centrifuged most easily.

Fig. 2. (Top). Extraction of trehalose by aqueous TCA (○), ethanolic TCA (×), and Cetavlon (●). (Bottom). Extraction of RNA by cold M-NaOH. ○, RNA pentose; ×, hexose in extract.

Extraction of nucleic acid by alkali from TCA-extracted (trehalose-free) yeast. The Schmidt-Thannhauser procedure (which has been applied to yeast by Bourdet *et al.* 1951) was found to extract considerable amounts of mannan and glycogen, together with RNA. Long contact with alkali to hydrolyse RNA to nucleotides was found unnecessary. The extraction step alone has been reported to require a much shorter time (DiCarlo & Schultz, 1948), and indeed RNA was rapidly extracted by shaking 200 mg. of trehalose-free yeast with 1 ml. of N-NaOH at room temperature (Fig. 2); 30 min. was considered adequate, subsequent increase of colour in the orcinol estimation being due mainly to polysaccharide. At this time about 5% of total yeast polysaccharide had been taken out.

Extraction of nucleic acid by hot TCA or perchloric acid from trehalose-free yeast. The Schneider technique (TCA for 15 min. at 90°) partially extracted mannan and glycogen together with RNA, in amounts sufficient to interfere with the pentose determination. That the increase in 'pentose' with extraction time (Fig. 3) was due to polysaccharide was indicated by the linear relation between optical density at 660 m μ . in the orcinol reaction and the total carbohydrate in the extracts as determined with anthrone, and by the increasing ratio (optical density at 420 m μ .)/(optical density at 660 m μ .) in the orcinol procedure (cf. Drury 1948). Extrapolation of the apparent RNA to zero extraction time gave a figure close to that obtained by alkali extraction. Substitution of

perchloric acid for TCA increased the rate of polysaccharide extraction (Fig. 3).

Fig. 4 shows the effect of (a) perchloric acid concentration, and (b) temperature on the amount of polysaccharide and RNA pentose extracted after 15 min. It appeared from these results that the best method of reducing contamination of RNA by polysaccharide was to use a low extraction temperature. At the convenient temperature of 38° the time of extraction had to be extended to 90 min. (Fig. 5).

About 1% of the RNA remained in the cells after treatment with 0.5M perchloric acid for 90 min. at 38°, as was shown by further extracting the cell residue with TCA at 90° for 15 min. and estimating pentose and hexose in the TCA extract by the orcinol method of Drury (1948).

Loss of RNA during the extraction of trehalose by cold TCA proved more serious. By studying the effect of time of extraction by TCA (at room temperature) on the subsequent analysis of RNA pentose in the cell residue, it was estimated that of the total RNA pentose, 6%/hr. had been lost during TCA extraction of trehalose and coenzymes.

Fig. 3. Extraction of RNA (●), glycogen (○) and mannan (×) by 0.5M TCA (full line) and of glycogen and mannan by 0.5M perchloric acid (broken line) at 90°, after trehalose extraction by cold TCA.

Trehalose extraction by TCA was therefore carried out at 0° in order to reduce to the minimum the loss of nucleic acid.

Extraction by alkali of a polysaccharide fraction rich in mannan, after removal of trehalose and nucleic acid. Digesting yeast (freed of trehalose) with 10M-KOH at 100° was found to cause severe loss of the total carbohydrate of the yeast suspension (Fig. 6), which paralleled the gradual solution of the cells. Use of hot, strong alkali as an extracting agent by Trevelyan & Harrison (1952) was therefore the probable cause of incomplete recovery (see Introduction). Milder extraction procedures were

Fig. 4. Effect of (a) acid strength (temp. 90°), and (b) temperature (acid 0.5M) on the extraction of RNA (○) and polysaccharide (×) by perchloric acid after trehalose extraction by cold TCA.

Fig. 5. Extraction of RNA (○) by 0.5M perchloric acid at 38° (after trehalose extraction by TCA). ×, Hexose extracted.

Fig. 6. Polysaccharide extraction. $\bigcirc$ — $\bigcirc$, Destruction of yeast polysaccharide by 10M-KOH at 100°; +, glycogen extracted by 2M acetic acid at 100° after trehalose and mannan extraction; $\times$, residual carbohydrate of cells after extraction by 0.5M perchloric acid at 100°; $\bullet$ — $\bullet$, extraction of alkali-soluble polysaccharide at 100° by 0.25M- Na_2CO_3 ; $\bigcirc$ — $\bigcirc$, extraction by 0.125M- Na_2CO_3 + 0.125M- NaHCO_3 .

examined. Mannan and a proportion of glycogen were readily extracted by shaking with 5M-KOH in the cold. Dilute alkali at higher temperatures was found more convenient, and similar results were given by 0.25M-NaOH at 90°, for 15 min. No more material was extracted when the time was extended to 60 min.

Subsequently the temperature of extraction was raised to 100° and the alkalinity of the reagents reduced still further by using mixtures of NaHCO_3 and Na_2CO_3 (each 0.25M, 4 ml. to 200 mg. of yeast). After 15 min., the following amounts of polysaccharide were extracted: 14% of total alkali-soluble polysaccharide by NaHCO_3 ; 25% by a 3:1 mixture of NaHCO_3 : Na_2CO_3 ; 43% by a 1:1 mixture; 92% by Na_2CO_3 . The last two reagents were further studied (Fig. 6). The standard procedure was to use 0.25M- Na_2CO_3 for 45 min. at 100° for the preparation of an alkali-soluble fraction, largely mannan.

The extract was partitioned into mannan and glycogen by precipitation of the mannan-copper complex. Flocculation of this after warming (Trevelyan & Harrison, 1952) was delayed when mild alkalis had been used to prepare the extract. Precipitation was therefore accomplished by leaving it overnight at room temperature (Chung & Nickerson, 1954). When extract obtained from 5 to 20 mg. of yeast was taken in a final volume of 6 ml. the mannan recovered was proportional to the

amount of yeast, showing that no significant loss had occurred during the precipitation.

Extraction of acid-soluble glycogen from the cell debris left after removal of mannan + alkali-soluble glycogen. Chung & Nickerson (1954) used 2M acetic acid (for 30 min. at 100°) to extract a second glycogen fraction. In the present work it was found that the carbohydrate extracted by this reagent was still increasing even after 2 hr., though it appeared to be approaching a limit of about 50% of the 'acid-soluble glycogen' estimated when m- H_2SO_4 was used (Trevelyan & Harrison, 1952). Moreover, after 2 hr. extraction by acetic acid the cell debris still gave a strong positive reaction with the periodic acid-Schiff stain, showing that some polysaccharide other than glucan remained (Trevelyan & Harrison, 1952). Perchloric acid (0.5M), on the other hand, gave a non-staining residue after extraction at 100° for 30 min. Also, the carbohydrate content of cells extracted by this reagent fell rapidly in the first 30 min., after which a very slow decrease occurred (Fig. 6). Yeast-glucan content was thus taken to be given by analysis of carbohydrate in the cells after 30 min. extraction. The interesting observation was made that when the cells had been heated with acid to the point where the periodic acid-Schiff stain became negative, the suspension flocculated on cooling, presumably reflecting some change in surface characteristics of the yeast cells.

Fractions obtained from larger quantities of yeast by successive extractions with acetic acid and sulphuric acid (which may be substituted for perchloric acid) were examined for reducing sugars by paper chromatography. Some glucose was detected in the second extract, and when this was further heated for 1 hr. at 100° more glucose appeared, together with a trace of mannose.

Changes during fermentation of yeast carbohydrate fractions, nucleic acid, and TCA-soluble and HClO_4 -soluble phosphate

More information on the significance of alkali-, acetic acid- and HClO_4 -soluble glycogen was obtained by following the change in yeast carbohydrate during anaerobic fermentation. Trehalose was extracted by TCA at room temperature, otherwise the analytical procedure was as described in Methods. Fermentation of glucose or mannose, in a medium lacking assimilable nitrogen, resulted in synthesis of mannan, glucan, and the three forms of glycogen (Fig. 7). Acetic acid-soluble glycogen showed the greatest increase. The changes in trehalose content under these conditions have been considered in a previous paper (Trevelyan & Harrison, 1956a). Uptake of phosphate from the medium into both the TCA- and HClO_4 -soluble fractions accompanied carbohydrate assimilation

Fig. 7. Polysaccharide metabolism during anaerobic fermentation at 30° by 2.5% (w/v) yeast. (a) N-free medium, 0.3M glucose; (b) as (a), +azide (mm-Na₃N₃); (c) full medium, 0.3M glucose; (d) N-free medium, 0.3M mannose; (e) N-free medium, 0.15M maltose. Full medium contained 0.05M sodium succinate buffer, pH 5.5, 5 mM-MgCl₂, 2 mM-KH₂PO₄, 8 mM-KCl, 0.01M-(NH₄)₂SO₄, 0.02M DL-asparagine and 5 μM thiamine. N-free medium had no asparagine or (NH₄)₂SO₄. O---O, mannan; x---x, glucan; O—O, alkali-soluble glycogen; ●, acetic acid-soluble glycogen; x—x, perchloric acid-soluble glycogen.

Fig. 8. Trehalose (●), nucleic acid (○), total acid-soluble phosphorus (+) and total phosphorus in nucleic acid extract (x) during fermentation under the conditions of Fig. 7.

(Fig. 8). RNA pentose showed little variation (Fig. 8), though later work (unpublished results of eighteen fermentations of glucose) shows that a slight but statistically significant increase in RNA pentose occurs during the first 30 min. Except where metaphosphate formation clearly accounted for an excess of phosphate, the RNA pentose figure agreed fairly well with the total HClO_4 -soluble phosphate. Addition of assimilable nitrogen to the medium restricted glycogen and trehalose formation without affecting the synthesis of mannan or glucan. RNA pentose increased in this case.

The inhibitor sodium azide prevented trehalose resynthesis (Trevelyan & Harrison, 1956a), and after a slight initial decrease in acetic acid-soluble glycogen the polysaccharides showed no further change. Phosphate uptake was inhibited.

Maltose is fermented more slowly than either glucose or mannose; the fermentation rate increases with time owing to adaptive-enzyme formation. During maltose fermentation in N-free medium (Figs. 7, 8) glycogen synthesis and phosphate uptake were observed only in the later stages of the fermentation.

Of the three glycogen fractions the alkali- and acetic acid-soluble glycogen varied together. Within the experimental error, one fraction was a simple proportion of the other; the regression equation

$$(\text{alkali-soluble glycogen}) = 3 + 0.41(\text{acetic acid-soluble glycogen})$$

had a residual error of estimate of $\pm 4 \mu\text{moles/g.}$ HClO_4 -soluble glycogen was not, however, simply related to the other fractions. Regression equations contained a large constant term, e.g. $(\text{HClO}_4\text{-soluble glycogen}) = 40 + 0.28(\text{acetic acid-soluble glycogen})$, error $\pm 10 \mu\text{moles/g.}$ This fraction could not be regarded statistically as a mixture of acetic acid-soluble glycogen and mannan or glucan. Mannan and glucan changed together, and the ratio mannan/glucan was approximately constant at 1.8.

The results showed (1) that nucleic acid could be separated with negligible loss of polysaccharide; the mean hexose content of the fraction was only $5.9 \pm 0.9 \mu\text{moles/g.}$ of yeast (s.d. twenty results); (2) that fractionation was accomplished without loss of material, since the mean difference (total carbohydrate) - (sum of fractions) was +10, with s.d. $\pm 11 \mu\text{moles/g.}$ on an average total of 420; (3) that no difficulty was experienced in centrifuging at any stage of the procedure, 5 min. at 4000 rev./min. being adequate. The main aims of the present study had, therefore, been achieved.

Influence of some constituents of the medium on the synthesis of yeast carbohydrates during anaerobic fermentation

Division of glycogen into subfractions did not seem justified by the results of the preceding section. The shortened fractionation scheme (see Methods), in which the acetic acid-extraction step was omitted, was therefore adopted as standard.

The procedure was evaluated by applying it to study the influence of constituents of the medium on carbohydrate synthesis during fermentation of glucose for 2 hr. The forty fractionations included ten performed on the yeast before fermentation, which gave a good idea of the reproducibility of the method (Table 1). These were done over a 27-day period, during which time the master sample of yeast metabolized part of its stores of trehalose and glycogen. Variance due to the linear regression was allowed for in calculating the errors listed in Table 1. It will be seen that estimations were reproducible to 3% or less; this figure included the sampling error.

The results showed an interaction between the effects of H_2PO_4^- and Mg^{2+} on glycogen and trehalose synthesis (Fig. 9). K^+ (0.02M-KCl), Mg^{2+} (0.01M- MgSO_4) and thiamine (50 μM) when added singly had only minor influence on changes in the various fractions. H_2PO_4^- , however, reduced glycogen formation and retarded the onset of the

Table 1. *Reproducibility of fractionation procedure*

T = Age of yeast in days (arbitrary zero).

Estimation	No. of samples	Mean ($\mu\text{moles of hexose, pentose or phosphate/g. of yeast}$)	S.D.
(Total carbohydrate) - (sum of fractions)	40	0.4	18
Total carbohydrate	10	$573 - 3.5T$	9
Trehalose	10	$123 - 1.2T$	2
Glycogen	10	$120 - 1.1T$	5
Hexose in RNA fraction	10	5.2	0.6
Mannan	10	195	6
Glucan	10	109	2
RNA pentose	7	41.3	0.5
HClO_4 -soluble P	10	40.6	0.7
TCA-soluble P	11	23.4	0.7

Fig. 9. Effect of some constituents of the medium on the synthesis of cellular carbohydrates during fermentation of 0.3M glucose at 30° by 2.5% (w/v) yeast. $\bigcirc-\bigcirc$, Trehalose; $\bullet-\bullet$, glycogen; $\square-\square$, mannan; $\times-\times$, glucan; $\times-\text{---}-\times$, total yeast carbohydrate. The yeast was suspended in 0.05M sodium succinate buffer, pH 5.5, with the following additions: (a) 0.02M-KCl; (b) 2 mM-KH₂PO₄ + 18 mM-KCl; (c) 0.02M-KH₂PO₄; (d) 0.01M-KH₂PO₄ + 5 mM-MgSO₄; (e) as (d), +0.05M-NH₄Cl; (f) 0.05M-NH₄Cl only.

Fig. 10. RNA pentose ($\bigcirc$), total acid-soluble phosphorus ($+$), and total phosphorus in RNA extract ($\times$) during fermentation under the conditions of Fig. 9.

formation of mannan and glucan (Fig. 9). Fermentation rate had been found (Trevelyan & Harrison, 1954) to be decreased by phosphate in the later stages of fermentation, an effect prevented by the simultaneous addition of Mg^{2+} . Here, too, Mg^{2+} removed the inhibitory action on glycogen synthesis, and more trehalose was formed than in succinate buffer alone. Mg^{2+} also stimulated phosphate uptake (Fig. 10).

In succinate buffer alone, or together with K^+ , Mg^{2+} , or thiamine (added singly), the RNA content of the yeast suspension fell slightly towards the end of the 2 hr. fermentation. $H_2PO_4^-$ or NH_4^+ singly prevented this fall, but with Mg^{2+} added as well as phosphate a distinct upward trend appeared (Fig. 10). This was naturally intensified by further supplementation with NH_4^+ .

NH_4^+ , added together with phosphate and Mg^{2+} , might almost be described as nullifying the effects of Mg^{2+} (Fig. 9). NH_4^+ alone reduced trehalose and glycogen formation, though not to the same degree (Fig. 9).

The restriction of carbohydrate formation by NH_4^+ was implicit in the finding by Meyerhof & Schulz (1936) that addition of ammonium salts increased the yield of CO_2 recorded during complete fermentation of glucose or fructose. They reported a similar, but smaller, increase when fermentation was carried out in an atmosphere of CO_2 (replacing N_2). The effect of this substitution on carbohydrate synthesis during fermentation in succinate buffer supplemented with Mg^{2+} and KH_2PO_4 was found to be slight, and results are consequently not reported in detail.

DISCUSSION

Although the fractionation procedure described by Trevelyan & Harrison (1952) has been considerably simplified and speeded, the division of polysaccharides into glycogen, mannan, and glucan remains on a semi-empirical basis. It is not, however, entirely arbitrary, as is shown by the changes in the various fractions during fermentation. Mannan and glucan are synthesized in parallel, as would be expected since both are constituents of the cell wall. Their formation is not reduced by nitrogen assimilation, as is the formation of trehalose and glycogen. (Since cell division entails the expansion of the total cell surface in a culture, the result is not unexpected; but the apparent lack of interaction between the synthetic mechanisms of the two groups of carbohydrates raises interesting problems.) As far as physiological behaviour goes, there would seem to be no great overlap between the various fractions.

The material designated 'glycogen' is clearly heterogeneous, physiologically if not chemically.

Glycogen, unlike mannan or glucan, is not confined to the cell wall (Northcote, 1953), and its occurrence in different regions of the cell may mean that glycogen is not uniformly free to diffuse out of the cell when yeast is treated with reagents such as carbonate or acetic acid. Variation in the extent of binding with protein, etc., may also be expected.

The effect on carbohydrate synthesis during fermentation of KH_2PO_4 may be due to formation of a complex of Mg^{2+} with metaphosphate formed during phosphate uptake. As Mg^{2+} participates in most enzyme systems concerned with phosphorylation processes, the mechanism of this effect may be extremely complex.

SUMMARY

1. A rapid method is described for adjusting the water content of the anthrone reagent to ensure that carbohydrate determination by the heat-of-mixing technique is carried out at an optimum temperature for the particular sugar concerned.

2. Methods are examined for partition of yeast carbohydrate into trehalose, mannan, glycogen and glucan, and for extracting nucleic acid free from polysaccharide.

3. By using the fractionation scheme proposed, and determining carbohydrate by the anthrone method and nucleic acid by the orcinol method, the separate fractions can be estimated to better than $\pm 3\%$. An internal check is provided by the agreement between the sum of the carbohydrate fractions and the directly determined figure for total yeast carbohydrate.

4. By the use of the scheme, it is shown that nitrogen assimilation restricts synthesis, during anaerobic fermentation of glucose, of the energy-reserve carbohydrates trehalose and glycogen, but not of the structural carbohydrates mannan and glucan. Carbohydrate formation is reduced also by high concentrations of phosphate, an effect which is counteracted by magnesium ions.

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A Study of the Nature of Plant Lignin by Means of Alkaline Nitrobenzene Oxidation

BY F. E. ROADHOUSE AND D. MACDOUGALL
 Ontario Agricultural College, Guelph, Canada

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Studies on the development and nature of lignin in plant tissues have been greatly hampered by the limitations of existing methods for its determination. The difficulty encountered is due to inadequate means for the characterization of lignin and for its isolation from other plant constituents. Two types of error may occur during the separation of lignin: losses during the pretreatment and isolation procedures, and contamination with interfering material arising from the action of the extracting reagents on other plant constituents. It is generally conceded that the advantages of the pretreatment extractions outweigh the disadvantages. Furthermore, the nature of both the lignin and the potential interfering substances changes with increasing age, so that a procedure which is adequate at one growth stage may be quite inadequate for some other growth stage.

In order to evaluate the various errors in the problem outlined above and, at the same time, to obtain more reliable information on the formation of lignin in plants, it was decided to oxidize plant materials of different species and ages with alkaline nitrobenzene and to determine the amounts of vanillin, *p*-hydroxybenzaldehyde and syringaldehyde formed. An attempt was made to determine lignin losses due to the pretreatment extractions with ethanol-benzene, water, and 1% hydrochloric acid by subjecting the residues from each step to alkaline nitrobenzene oxidation.

The alkaline nitrobenzene technique was used by Freudenberg, Lautsch & Engler (1940). These workers found that sulphonated lignins, isolated

lignins, and wood itself gave substantial yields of vanillin when oxidized with this reagent. Creighton, McCarthy & Hibbert (1941) applied the technique to a large number of materials (jute, rye straw, corncobs, bamboo, sassafras, etc.). They obtained sufficient vanillin and syringaldehyde from angiosperms to account for 25-45% of the Klason lignin. In later work (Creighton & Hibbert, 1944) vanillin and syringaldehyde were isolated after oxidation of dicotyledonous plants. From monocotyledons *p*-hydroxybenzaldehyde was obtained in addition to the other two aldehydes. Bondi & Meyer (1948) used the same techniques to investigate the lignins from grasses, legumes and sheep faeces.

More recently, Stone & Blundell (1951) have described a micromethod for alkaline nitrobenzene oxidation. Stone, Blundell & Tanner (1951) used this technique on wheat plants and observed that the ethanol-benzene extraction removed appreciable amounts of lignin, the water extraction removed traces of lignin, and the 1% hydrochloric acid treatment removed little or no lignin. They also reported that the decreasing order of abundance of the aldehydes in young tissues was *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde. In the mature plant this order was reversed.

MATERIALS AND METHODS

Plant tissues. The plants used in this investigation were winter wheat (Dawson's Golden Chaff, no. 16), alfalfa (O.A.C. no. 1), and meadow fescue (Mefon). These were