SUMMARY

1. A preparation of completely haemolysed erythrocytes from the blood of domestic fowls, which actively incorporates [α - ^{14}C]glycine into haem, is described.

2. The effects of time and of glycine concentration on the rate of synthesis were studied.

3. Stimulation of the synthesis up to sixfold was obtained with some preparations of boiled yeast extract. 2:4-Dinitrophenol inhibited the synthesis almost completely at $5 \times 10^{-4}\text{M}$.

4. Synthesis of free protoporphyrin also occurs during incubation of the haemolysate.

5. Contrary to the findings of Gajdos & Gajdos-Török (1951), using the blood of phenylhydrazine-anaemic rabbits, sodium fluoride was found to inhibit free porphyrin synthesis both in haemolysates and in whole blood of fowls.

6. The effects of various other substances, including some erythropoietic agents, are also described.

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Synthesis of Cellulose by *Acetobacter xylinum*

1. MICROMETHOD FOR THE DETERMINATION OF CELLULOSES*

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The use of micro-organisms in studies of cellulose synthesis is dependent on the development of suitable methods for the quantitative determination of cellulose in small samples. For this purpose, several types of approach might be considered; more especially, methods based on: (i) the colour reactions shown by carbohydrate on heating with a chromogen in the presence of strong acid, (ii) total combustion, and (iii) reductometry of a hydrolysate. In view of the relative specificity and precision of reductometric methods of sugar assay, it seemed preferable in principle to base the micromethod for cellulose on the last of the general possibilities listed above. However, the direct quantitative conversion

of cellulose into glucose by acid hydrolysis under conditions tolerated by glucose requires many hours (cf. Heuser, 1946). It will be shown in this communication that the difficulty inherent in the direct hydrolysis of cellulose fibre can be avoided if the material is first subjected to acetolytic degradation. With this means the fibre is rapidly made soluble and a product is obtained which can be hydrolysed quantitatively with relative ease. A microprocedure for the estimation of cellulose has been based on this principle.

The method detailed below has been applied extensively only to the study of the formation of cellulose by washed cell suspensions of *Acetobacter xylinum* in glucose solution (Hestrin, Aschner & Mager, 1947). It is anticipated that the procedure will also prove suitable for use with other celluloses.

* Experiments presented in this communication were performed by one of us (M.S.) in part fulfilment of requirements for a Ph.D. degree.

It may have advantages, moreover, for determination of certain of the difficulty hydrolysable non-cellulosic polysaccharides, e.g. dextrans. The applicability to cotton cellulose as well as to bacterial cellulose has been verified.

MATERIALS AND METHOD

Reagents

Sodium hydroxide, 8 and 16 % (w/v).

Talcum powder.

Acetolytic reagent. Acetic anhydride, glacial acetic acid and sulphuric acid, sp.gr. 1.84 (1:1:0.05, v/v). This reagent is prepared immediately before use.

Hydrolytic reagent. Sulphuric acid (sp.gr. 1.84) and water (15:85, v/v).

Copper reagent. The highly alkaline copper reagent of Somogyi (1945) was used with a minor modification. To avoid autoreduction during storage, the CuSO_4 is stored separately in aqueous solution and added to the stock solution of the other required components on the day the reagent is used.

Arsenomolybdate solution. This was prepared as described by Nelson (1944).

Sulphuric acid reagent. Sulphuric acid (sp.gr. 1.84) and water (21:479, v/v).

Reference sample. Samples of pellicles from 7-day glucose cultures were rinsed, pressed out repeatedly in water, and treated with 1 % (w/v) NaOH at room temperature for 1 hr. and at 98° for 5 min. to dissolve the bacterial cells. The product, after further repeated rinsing and pressing in water, was dried *in vacuo* at 90°. (Found: ash, 1.0; N (by Kjeldal), 0.3, moisture, 3.6 %.)

Analytical procedure

Preparation of sample for analysis. The glassware should be scrupulously protected from contamination by airborne cellulosic dusts. The suspension of cells of *A. xylinum* with the formed cellulose floccules and pellicle is washed into a centrifuge tube which has a mark at 10 ml. The insoluble particles are packed by centrifuging (about 4000 rev./min. for 15 min.). Since, at this stage, the cellulose is accompanied by the cells which it enmeshes, it settles readily in the centrifugal field. In absence of weighting protein, or if entrained air bubbles are present, sedimentation may be facilitated by addition of a pinch of talcum powder and/or a little ether. The precipitate is freed from the water-soluble residues by resuspension in water and again packed by centrifuging. To the precipitate is added 1 ml. of water. Large clumps are broken by shaking. To the suspension, 1 ml. of 8 % (w/v) NaOH solution is added to give a final concentration of 4 %. To make the cells soluble, the suspension is held in a boiling-water bath for 5 min., the tube being shaken briefly after 2 or 3 min. to facilitate dispersion of the solids. Since the alkali-purified cellulose cannot be sedimented readily, a pinch of talcum powder is added to provide an effective weighting agent. In rare instances in which trapped air bubbles cause flotation, a glass rod shaken in the suspension serves to dislodge the troublesome bubbles. The volume is increased by addition of water, and the cellulosic material with the enmeshed talcum powder is packed tightly by centrifuging (about 6000 rev./min. for 5 min.). To remove residual alkali-soluble material and to lower the NaOH concentration, the precipitate is suspended

in water and again packed by centrifuging. Finally, the tube is thoroughly drained and held at 80–90° until the material appears to be dry.

Determination of the cellulose content of the purified cellulosic fraction. The analytical procedure comprises three steps: solution of the purified fraction by acetolysis, acid hydrolysis of the product so obtained, and estimation of the liberated reducing sugar.

Acetolysis. To the dried and purified cellulosic fraction (50–5000 μg .) contained in a tube with a mark at 10 ml. is added 1 ml. of the acetolytic reagent. If the sample contains less than 50 μg . cellulose, use of a smaller volume of the acetolysing reagent is advantageous. The volumes of reagents added subsequently must then be reduced proportionately. The tube is covered with a glass bulb, transferred to a boiling-water bath, and shaken briefly at the beginning of the reaction to facilitate solution. After 20 min., the tube is removed from the bath and allowed to cool. A colourless or faintly amber solution is obtained. The particles of talcum powder do not need to be removed at this stage.

Hydrolysis. To the acetolysed cellulose solution (1 ml.) is added 1 ml. of the hydrolytic reagent and the fluids are mixed by shaking. The solution may become turbid at this stage if the cellulose content is high. The hydrolysis is conducted under a glass bulb for 40 min. in a boiling-water bath. The tube is then transferred to an ice bath and the contents are neutralized by addition of a suitable volume of 16 % NaOH solution. The volume of alkali solution required is determined beforehand on a blank which contains the same reagents without cellulose, phenolphthalein serving as the indicator. A small excess of alkali is difficult to avoid, but the loss of sugar from this cause is negligible when the temperature is kept low by the use of an ice bath. The solutions, immediately on the removal of each tube from the ice bath, are brought to the mark (10 ml.) by addition of water and mixed thoroughly with the aid of a pipette. The talcum powder present is removed. The reducing power is determined on a suitable sample not later than 1 hr. after completion of the hydrolysis.

Determination of reducing power. The copper reagent (2 ml.) and the sample of the cellulose hydrolysate are mixed, brought to a volume of 4 ml., and heated for 25 min. in a boiling-water bath. To the cooled solution is added 2 ml. of the arsenomolybdate solution, followed by sufficient of the sulphuric acid reagent (1 ml. reagent/ml. sample) to overcome the buffering power of the sodium acetate present in the sample. The solution is swirled, made up after 5 min. with water to 10 ml. and the colour read. Filter 54 (540 $\text{m}\mu$.) is most satisfactory in the Klett photoelectric colorimeter. Filter 66 (660 $\text{m}\mu$.) affords increased sensitivity and can be used when very small samples are to be determined. The measuring instrument is set to zero against a reagent blank which has been taken through the same procedure.

The values are read from a standard calibration curve obtained with glucose in water using the standard conditions. With a Klett photoelectric colorimeter as the measuring instrument, the lower threshold of the method is about 20 μg . The readings taken from the glucose calibration curve correspond without further correction to cellulose. Since 162 g. cellulose are equivalent to 180 g. glucose, the weight of glucose found should be multiplied by 0.9. However, as mentioned below, only 90 % of the bacterial or cotton cellulose is recovered as glucose. Consequently, when corrected for this loss, the overall conversion factor of glucose to cellulose is unity.

EXPERIMENTAL AND RESULTS

Alkali purification of the samples of bacterial cellulose

An alkaline reagent was desirable which would remove the non-cellulosic components promptly and with high degree of selectivity. It was found that treatment for 5 min. in 4% (w/v) sodium hydroxide at 97° fulfils this requirement. The insolubility of the cellulose of *A. xylinum* under these conditions was demonstrated by the finding that the analytical yield of cellulose was not increased when the concentration of sodium hydroxide was decreased from 4 to 1%.

Course of hydrolysis of acetolysed cellulose

The marked effect of primary acetolytic degradation on the course of the subsequent acid hydrolysis of the cellulose is shown by the experiment summarized in Fig. 1. When the preliminary acetolytic treatment was omitted, the recovery of reducing sugar even after 4 hr. hydrolysis was only about 5%. It is apparent that the hydrolysis of the acetolysed cellulose is completed under standard conditions within about 40 min., that the yield of reducing sugar at that time does not depend critically on the hydrolysis time, and that the recovery corresponds to 90% of the theoretical glucose yield. The shape of the hydrolysis curve of the acetolysed cellulose, and the observation reported later that glucose slowly loses reducing power under these conditions do not encourage the hope that a longer hydrolysis time would lead to an increased yield of reducing sugar beyond the observed maximum value.

An experiment to check whether the percentage yield of the reducing sugar under the conditions of the analysis is variable with sample weight is shown in Fig. 2. The points for both the *A. xylinum* cellulose and raw cotton cellulose fall on the same straight line which passes through the origin. The percentage yield of sugar is clearly independent of the weight of the cellulose sample within a wide range.

Applicability of the procedure to celluloses from different sources

It has been shown that the method is applicable to bacterial celluloses, both to the cellulose formed by *A. acetigenum* and to that formed by *A. xylinum*. The applicability to cotton cellulose has been verified with raw cotton samples taken from two locations and on a sample of cotton yarn containing 95% α -cellulose and 5% γ -cellulose. The recovery of reducing sugar under standard conditions in the cases listed above averaged 90% of the theoretical, the scatter of the results around this mean being within the range $\pm 4\%$. In four analyses on filter paper, the recovery of glucose was $92 \pm 4\%$.

Fig. 1. Hydrolysis of bacterial cellulose. Samples of bacterial cellulose (~5 mg.) were weighed on the semi-microbalance. Reagent compositions and temperatures as described for the standard procedure. ●, cellulose after previous acetolysis; hydrolysis reagent added at zero min. ○, cellulose without previous acetolysis; hydrolysis reagent and acetolysis reagent added simultaneously at zero min.

Fig. 2. Influence of weight of cellulose on sugar recovery. Samples of purified fibre were weighed on a semi-microbalance accurate to 0.02 mg. Cellulose weights are corrected for moisture, ash and protein. Standard analytical procedure. ●, raw cotton (Egyptian); ○, *A. xylinum* cellulose.

When a sample of pure glucose was taken through the procedures described for the cellulose analysis, the reducing power was 80% of the original value, this value being independent of the duration of heating in the hydrolysis reagent between 0 and 50 min. The observed disparity between the behaviour of glucose and cellulose may be due to the presence of a free reducing group in the glucose. When glucose was exposed directly to the action of the hydrolysis reagent in the presence of the acetylating reagent, a slow loss of reducing power occurred

with continued heating, the reducing power after 0, 40 and 50 min. of heating being 100, 91 and 89 % respectively.

The findings with filter paper and glucose suggest that the conditions of the analysis, as adapted to bacterial cellulose, can be used with cellulose polymers of widely varying degree of polymerization. Although some loss of reducing power due to the instability of a free reducing group may be expected when the analysis is applied to the lower homologues, it is evident that, even in the extreme case of glucose, the overall loss of reducing power is not greater than 20 %.

*Influence of salt concentration on
colour intensity*

The neutralized cellulose hydrolysate contains approximately 1.7 m-moles/ml. sodium acetate and 0.3 m-mole/ml. sodium sulphate. The colour formed with the arsenomolybdate reagent was found to be unaffected by the presence of these salts, provided that the buffering action of the sodium acetate was overcome by an appropriate addition of acid.

SUMMARY

1. A technique for the purification and quantitative recovery of small samples of bacterial cellulose has been detailed.

2. A micromethod for cellulose has been based on the dissolution of the purified polysaccharide by acetolytic degradation, the conversion of the product into reducing hexose by acid hydrolysis, and the colorimetric estimation of the formed sugar with the aid of a copper reagent.

3. The applicability of the technique to bacterial celluloses and cotton cellulose has been verified.

4. The method is relatively rapid and specific and is appropriate to serial analysis. The lower threshold is 20 μ g.; the error range is ± 4 %.

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Biochemical Studies of Toxic Agents

5. OBSERVATIONS ON THE FATE OF ^{35}S -LABELLED ARYLSULPHURIC ACIDS FOLLOWING THEIR ADMINISTRATION TO THE RAT

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A number of workers (for example, Rhode, 1923; Sperber, 1948; Garton & Williams, 1949) have studied the fate of arylsulphuric acids in the animal body, and their results suggest that when these compounds are administered to animals almost the whole of the dose is excreted unchanged in the urine. These findings were based on the determination of the increase in ethereal sulphate excretion over the 'normal level' following the administration of the sulphuric acid derivative. As ethereal sulphate excretion fluctuates somewhat under normal conditions, it is clear that experiments of this type cannot be expected to reveal with certainty a small degree of hydrolysis of an arylsulphuric acid *in vivo*. A more satisfactory method for studying this problem is provided by the measurement of the excretion of ^{35}S -labelled inorganic sulphate following the administration of an arylsulphuric acid labelled with ^{35}S . This method has been used in the present investigation. The potassium salts of [^{35}S]phenylsulphuric acid, and [^{35}S]1- and [^{35}S]2-naphthyl-

sulphuric acid were synthesized and administered to rats by stomach tube and by intraperitoneal injection. The urinary excretions of inorganic sulphate- ^{35}S and total sulphate- ^{35}S were determined at various times during the 96 hr. after dosing. Similar results were obtained with the three compounds, namely, that although most of the administered arylsulphuric acid was quickly excreted unchanged, some breakdown of the compound did occur in the organism.

EXPERIMENTAL

Synthesis of the potassium salts of phenylsulphuric acid and 1- and 2-naphthylsulphuric acid labelled with ^{35}S

The method used for the synthesis of the radioactive arylsulphuric acids was based on that described by Burkhardt & Lapworth (1926). Except in the final stages, the same procedure was used for the preparation of all three compounds. The [^{35}S]chlorosulphonic acid used in the syntheses was obtained from the Radiochemical Centre, Amersham. It was