A Micro-determination of Carbohydrates and Proteins

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Johnson (1949) described a method for estimating some proteins and carbohydrates in aqueous solution and suggested the use of the method in assessing the purity of enzymic fractions. He pointed out that biological activities quoted in terms of dry weight are less useful than those given in terms of organic material, particularly when inorganic salts are used in purification procedures. Furthermore, large samples are required to avoid errors in weighing. Dixon & Webb (1958) have also suggested the use of the method to follow enzymic purifications. Johnson's (1949) procedure uses a mixture of sodium dichromate in concentrated sulphuric acid to oxidize the sample (100–500 $\mu\text{g.}$ in 0.4 ml.), after which the excess of dichromate is determined colorimetrically. The method has been applied by Halliwell (1958) to the determination of insoluble carbohydrate in the form of cellulose. Johnson's method (1949) for soluble carbohydrates and proteins is both rapid and useful, except for the amount of organic material required, particularly in enzymic work. We have developed a procedure in which only 9–40 $\mu\text{g.}$ of carbohydrate or protein in up to 0.45 ml. is used. A submicro-procedure is also described for carbohydrates in amounts of 0.9–4 $\mu\text{g.}$ by using one-tenth the volumes of reagents and sample. These procedures involve reaction of the excess of dichromate with starch-iodide reagent. The intensity of the blue colour is measured with a photoelectric absorptiometer. The original procedure of Johnson (1949) has also been modified and made more reliable for the range 100–700 $\mu\text{g.}$ of soluble organic material by the use of more dilute dichromate- H_2SO_4 solution and by using aqueous rather than solid sulphite. It has thus been possible to obtain the theoretical calibration curves (for carbohydrates) in practice.

The new procedure is recommended for the determination of carbohydrates, proteins and their hydrolysis or breakdown products formed enzymically or otherwise, for following the purity of proteins or enzymes during fractionation procedures and for the analysis of chromatographic eluates.

MATERIALS AND METHODS

General reagents

Standard solutions of carbohydrates and proteins. Glucose (A.R.), sucrose (A.R.) cellobiose (Light and Co. Ltd.),

soluble starch (A.R.), crystalline samples of pepsin, trypsin and bovine plasma albumin (Armour Laboratories), Bacto-peptone and -tryptone (Difco Laboratories) were used. The products were analysed for moisture (dried at 105°) and ash content (750° for 3 hr.) and where necessary the figures quoted in the text are corrected for these values. The carbohydrates and proteins were prepared in aqueous solution at a concentration of approximately 40 $\mu\text{g.}$ in 0.45 ml.

Reagents for the determination of 9–40 $\mu\text{g.}$ of organic material in 0.45 ml. and for 0.9–4 $\mu\text{g.}$ in 0.045 ml.

Aqueous 3.4% (w/v) potassium dichromate. Potassium dichromate (A.R.) (1.7 g.) is dissolved in water with heating and the solution made up to 50 ml. This solution is stable for at least 6 months at room temperature in an all-glass vessel.

Solution (0.034%, w/v) of potassium dichromate in 97% (w/v) sulphuric acid. The 3.4% (w/v) solution (0.5 ml.) of $\text{K}_2\text{Cr}_2\text{O}_7$ in water is cooled in a vessel having a ground-glass neck and 49.5 ml. (50 ml. measuring cylinder) of 98% H_2SO_4 (Micro Analytical Reagent, British Drug Houses Ltd.) is added and mixed with further cooling. This reagent is prepared just before use.

Cadmium iodide-starch reagent. Cadmium iodide (A.R.) (1.1 g.) is dissolved in 34 ml. of water and the solution boiled gently (to remove possible traces of free I_2) for 15 min., the volume being kept constant. Meanwhile starch solution is prepared by boiling 0.26 g. of soluble starch (A.R.) in 46 ml. of water for about 5 min. until the liquid clears. This solution is then added slowly to the gently boiling CdI_2 solution and the mixture boiled for about 5 min. It is then filtered through a no. 3 sintered-glass filter (or no. 50 Whatman filter paper), cooled to room temperature and made up to 100 ml. with water. The reagent is stable for at least a month (see below) in clear glass-stoppered Pyrex flasks at room temperature out of direct sunlight.

Reagents for the determination of 100–700 $\mu\text{g.}$ of organic material in 0.9 ml.

Solution (0.25%, w/v) of potassium dichromate in 96% (w/v) sulphuric acid. Potassium dichromate (A.R.) (2.5 g.) is dissolved with heating in 20 ml. of water, and the solution cooled and diluted to 1 l. with 98% H_2SO_4 . The reagent is stored in a glass-stoppered vessel protected from dust and is stable at room temperature for at least 3 months.

Aqueous 20% (w/v) sodium sulphite. A solution of 4 g. of $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ (A.R.) in 20 ml. of water is prepared and stored as described previously (Halliwell, 1958). It is then stable for at least 2 weeks. The stability is readily checked by mixing 0.1 ml. of 20% (w/v) Na_2SO_3 solution with 2 ml. of 0.25% (w/v) solution of $\text{K}_2\text{Cr}_2\text{O}_7$ in 96% H_2SO_4 (see above) and 6 ml. of water. The colour produced is compared in an absorptiometer (Ilford violet filters no. 601, peak transmission 430 m μ) with that of a similar solution prepared in the absence of sulphite (see 'Analytical Procedure').

Analytical procedure

Colour measurement: preparation of calibration curves for 9–41 $\mu\text{g.}$ of standard solutions of carbohydrates in up to 0.45 ml. of solution by using the Spekker absorptiometer (Hilger). Suitable volumes to cover the range 0 (blank tube)—41 $\mu\text{g.}$ of each of the carbohydrates and proteins (including peptone and tryptone) are pipetted into tubes and all volumes made up to 0.45 ml. with water. The blank tube is required for $\text{K}_2\text{Cr}_2\text{O}_7$ in the absence of organic material. A 0.034% (w/v) solution (1 ml.) of $\text{K}_2\text{Cr}_2\text{O}_7$ in 97% H_2SO_4 is added and the reactants are mixed carefully. The tubes are closed with pear bulbs and heated in a boiling-water bath for 7 min. (carbohydrates) or 20 min. (proteins) and then cooled in cold water. Water (10 ml.) is added to each tube, the contents are mixed and 0.5 ml. is removed into 8.5 ml. of 0.21 N- H_2SO_4 in Quickfit test tubes, followed by the addition of 1 ml. of CdI_2 -starch reagent. The tubes are closed with glass stoppers and mixed by inversion so that the solution touches all the internal surface of the tube. The tubes are left at room temperature (19–25°) out of direct sunlight (complete darkness is not required) for 20 min. for development of the blue colour. The solutions are read against water in absorptiometer cells with a 1 cm. light-path by using Ilford orange filters (no. 607, peak transmission 600 m μ). A reagent blank (without $\text{K}_2\text{Cr}_2\text{O}_7$), which does not need boiling, is prepared by adding 0.5 ml. of 8.6% H_2SO_4 to 8.5 ml. of 0.21 N- H_2SO_4 , followed by 1 ml. of CdI_2 -starch reagent. [The 8.6% H_2SO_4 is prepared by diluting 1 ml. of 98% H_2SO_4 with 10.45 ml. of water to correspond with the acid concentration in the 0.034% solution of $\text{K}_2\text{Cr}_2\text{O}_7$ in 97% H_2SO_4 after dilution of the latter with the sample volume (0.45 ml.) and 10 ml. of water.]

A 2 ml. pipette may be used to deliver the 1 ml. of the 0.034% solution of $\text{K}_2\text{Cr}_2\text{O}_7$ in 97% H_2SO_4 and should have a drawn-out tip with an orifice about 1 mm. in diameter. In all stages before boiling the samples with $\text{K}_2\text{Cr}_2\text{O}_7$ - H_2SO_4 , glass paper (Whatman) is used to wipe off excess of sample and $\text{K}_2\text{Cr}_2\text{O}_7$ - H_2SO_4 from the tips of pipettes.

Submicro-determination of 0.9–4 $\mu\text{g.}$ of carbohydrates in 0.045 ml. The procedure is essentially the same as that described above except that one-tenth of the volumes of reagents are used. After heating and cooling, the tubes are diluted with 1 ml. of water and then washed out by decantation with a total of 20 ml. of 0.21 N- H_2SO_4 into larger tubes: 2.3 ml. of CdI_2 -starch reagent is added, the solutions are mixed and the colour is developed for 20 min. and read as described above.

Determination of 100–700 $\mu\text{g.}$ of carbohydrates and proteins. This procedure follows that of Johnson (1949), with slight modifications of reagents and with further application to carbohydrates. Briefly, suitable volumes of carbohydrates and proteins containing 0 (two blank tubes are required)—700 $\mu\text{g.}$ of material in 0.9 ml. of solution in Quickfit test tubes are treated with 2 ml. of a 0.25% solution of $\text{K}_2\text{Cr}_2\text{O}_7$ in 96% H_2SO_4 and heated in a boiling-water bath for 20 min. After cooling, and addition of 6 ml. of water and mixing, 0.1 ml. of aqueous 20% (w/v) $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ is used to reduce the $\text{K}_2\text{Cr}_2\text{O}_7$ in one of the two blanks. All tubes are then read against the reduced blank with violet filters (Ilford no. 601) as above.

RESULTS

Quantitative reaction of potassium dichromate in aqueous solution and in 97% H_2SO_4 with the cadmium

iodide-starch reagent. Before the determination of organic compounds by measuring an excess of unchanged dichromate was attempted it was first necessary to show the quantitative nature of the reaction of $\text{K}_2\text{Cr}_2\text{O}_7$ with starch-iodide reagent. The stock 3.43% (w/v) aqueous $\text{K}_2\text{Cr}_2\text{O}_7$ was diluted to 0.003% (w/v) with water. Portions (0–0.5 ml.) were then diluted to 9 ml. by addition of 2.2 ml. of 1.63 N- H_2SO_4 and water. Cadmium iodide-starch reagent (1 ml.) was added and the blue colours were developed for 20 min. and read as described under 'Analytical Procedure'. Fig. 1 illustrates the type of curve obtained. The final concentration of $\text{K}_2\text{Cr}_2\text{O}_7$ covered the range 0.00015–0.00015% (w/v). The final concentration of H_2SO_4 in the 10 ml. volume was about 0.36 N and gave a pH of 0.76. This concentration of acid was chosen after preliminary experiments with a range of acid concentrations had shown that the resultant pH was the most suitable for development of the blue colour. At the same time it was shown that the colour

Fig. 1. A. Calibration curve for the reaction of $\text{K}_2\text{Cr}_2\text{O}_7$ with CdI_2 -starch reagent. Aqueous $\text{K}_2\text{Cr}_2\text{O}_7$ (0–0.5 ml. of 0.003%, w/v) was added to 2.2 ml. of 1.63 N- H_2SO_4 and water in a final volume of 9 ml., followed by 1 ml. of CdI_2 -starch reagent. Colours were read as described in the text. B. Standard calibration curve for the determination of excess of $\text{K}_2\text{Cr}_2\text{O}_7$ by the iodometric micro-procedure after oxidation of organic material (glucose 9–41 $\mu\text{g.}$, in this case) by 1 ml. of a 0.034% solution of $\text{K}_2\text{Cr}_2\text{O}_7$ in 97% H_2SO_4 .

developed as well at 25° as at 16 ± 3°. All colours were read against water about 20 min. after mixing the $K_2Cr_2O_7$ solutions with CdI_2 -starch reagent. A reagent blank containing all solutions except $K_2Cr_2O_7$ was included. All readings increased slightly during the next 30 min. (total colour development time of 50 min.), the increase on the maximum reading being about 3%. Readings then remained constant for a further 25 min. (total colour development time of 75 min.) with no sign of precipitation.

Having shown the quantitative response of aqueous $K_2Cr_2O_7$ with starch-iodide reagent we then attempted a similar demonstration with a 0.034% solution of $K_2Cr_2O_7$ (w/v) in 97% H_2SO_4 the reagent used for oxidation of carbohydrates and

proteins. The acid dichromate was diluted with 10.45 vol. of water to give 0.003% (w/v) $K_2Cr_2O_7$ in acid. Portions (0.0–0.5 ml.) were added to 8.5 ml. of 0.21N- H_2SO_4 and sufficient 8.6% H_2SO_4 was added to give a total volume of 9 ml. (the 8.6% H_2SO_4 was prepared by diluting 1 vol. of 98% H_2SO_4 with 10.45 vol. of water). Cadmium iodide-starch reagent (1 ml.) was added and the blue colours were developed for 20 min. and read as described under 'Analytical Procedure'. A line similar to that of Fig. 1A was obtained, thus indicating that the linear response also applied to $K_2Cr_2O_7$ in conc. H_2SO_4 after suitable dilution. Thus it was now possible to examine the oxidation of micro-quantities of organic compounds with dichromate in conc. H_2SO_4 followed by iodometric determination of the excess of $K_2Cr_2O_7$. The method is described under 'Analytical Procedure' (see also Fig. 1B).

Determination of carbohydrates and proteins. Table 1 shows the amount of each compound equivalent to 1 ml. of the 0.034% solution of $K_2Cr_2O_7$ in 97% H_2SO_4 used in the oxidation. The equivalents were obtained by extrapolating to zero the calibration curves prepared for each compound in a manner similar to that for glucose of Fig. 1B. The method measures excess of $K_2Cr_2O_7$ present after oxidation of the organic compounds and the curve so obtained is in effect a 'colorimetric back titration'. Determination of glucose and starch in amounts of 0.9–4 μ g. by the submicro-procedure (see 'Analytical Procedure') gave similar curves. Determination of proteins in such small amounts (0.9–4 μ g.) was less successful, possibly owing to the longer heating period of 20 min.

Carbohydrates were also determined by the semi-micro-procedure with 100–700 μ g. of organic material in 0.9 ml. Equivalents were determined in a manner similar to that described above and are given in Table 2.

The theoretical reaction for oxidation of a monosaccharide, disaccharide and polysaccharide (we have used glucose, cellobiose and starch) may be represented thus:

from which it can be calculated that 1.177 g. of $K_2Cr_2O_7$ oxidize 180 mg. of glucose, 171 mg. of cellobiose or 162 mg. of starch. The ratio monosaccharide : disaccharide : polysaccharide is then 100 : 95 : 90. The theoretical equivalents in Tables 1 and 2 have been calculated from the above equation. Within experimental error the experimental values agree with the theoretical equivalents. Owing to the complex nature of the oxidation of proteins it does not appear possible to formulate simple theoretical equivalents, although the experimental values occur in a narrow range.

Table 1. Amounts of organic material found equivalent to potassium dichromate in sulphuric acid (iodometric micro-procedure)

Dichromate equivalents are the amounts (μ g.) of organic material oxidized by 1 ml. of a 0.034% (w/v) solution of $K_2Cr_2O_7$ in 97% (w/v) H_2SO_4 , as determined by the present iodometric micro-procedure with calibration curves prepared for each compound. The theoretical equivalent for the standardized $K_2Cr_2O_7$ in H_2SO_4 is 50 μ g. of glucose. Figures in parentheses under 'dichromate equivalents' are arbitrary values with glucose set at 100 (see text).

Organic material	Dichromate equiv. (mean value, μ g.)	Range of values obtained for dichromate equiv. (%)
Albumin	60	99–100
Trypsin	63	98–101
Pepsin	59	98–101
Tryptone	61	98–102
Peptone	62	99–101
Cellobiose	46 (94)	98–102
Starch	44 (90)	98–101
Glucose	49 (100)	98–101

Table 2. Amounts of organic material found equivalent to potassium dichromate in sulphuric acid (semimicro-procedure)

Dichromate equivalents are the amounts (μ g.) of organic material oxidized by 2 ml. of a 0.25% (w/v) solution of $K_2Cr_2O_7$ in 96% (w/v) H_2SO_4 determined by the semimicro-procedure of Johnson (1949) as modified under 'Analytical Procedure'. Equivalents are calculated from calibration curves prepared for each compound. Figures in parentheses under 'dichromate equivalents' are arbitrary values with glucose set at 100 (see text).

Organic compound	Dichromate equiv. (mean value, μ g.)	Range of values obtained for dichromate equiv. (%)	Theoretical value for dichromate equiv. (μ g.)
Cellobiose	725 (95)	100–101	724
Starch	687 (90)	100–101	688
Glucose	764 (100)	99–101	764

Recovery of cellobiose, sucrose and starch by using a glucose calibration curve. The carbohydrates were estimated by the iodometric micro-procedure in amounts of 10 and 40 $\mu\text{g.}$ in 0.45 ml. of solution. Recoveries were calculated from a standard glucose curve by using the factors 0.95 and 0.90 to convert the 'glucose' values of the determined disaccharides and polysaccharide into anhydroglucose values as described above. Table 3 indicates the recoveries obtained.

Stability of dilute potassium dichromate in 97% sulphuric acid. This reagent, prepared as under 'Analytical Procedure', was made up at three concentrations of dichromate at approximately 0.034% (w/v) (the concentration used in the present iodometric procedure), 0.017 and 0.003% (w/v) in 97% H_2SO_4 . Samples of each solution were heated in a boiling-water bath for periods up to 2 hr., cooled and compared with a corresponding sample of unheated solution of $\text{K}_2\text{Cr}_2\text{O}_7$, in 97% H_2SO_4 . Comparisons were made by the present iodometric micro-method on the absorptiometer. A sample of a 0.034% (w/v) $\text{K}_2\text{Cr}_2\text{O}_7$ in 97% H_2SO_4 was also examined for its stability at room temperature.

The 0.034% (w/v) solution in 97% H_2SO_4 represents the minimum concentration of $\text{K}_2\text{Cr}_2\text{O}_7$ in 97% H_2SO_4 suitable for general use in the oxidation of organic material. Smaller concentrations (0.017%, w/v) of $\text{K}_2\text{Cr}_2\text{O}_7$ in 97% H_2SO_4 are partly destroyed on boiling or completely destroyed [0.003% (w/v) $\text{K}_2\text{Cr}_2\text{O}_7$]. Even at room temperature (up to 20°) the 0.034% solution in 97% sulphuric acid loses some potency on storage for a few days. As a result it was found more convenient to prepare the 0.034% (w/v) solution of $\text{K}_2\text{Cr}_2\text{O}_7$ in 97% H_2SO_4 (the reagent used for oxidation of carbohydrates and proteins), when needed, by adding approx. 99 vol. of 98% H_2SO_4 to 1 vol. of 3.4% (w/v) aqueous $\text{K}_2\text{Cr}_2\text{O}_7$ (see 'Analytical Procedure'). The 3.4% (w/v) aqueous $\text{K}_2\text{Cr}_2\text{O}_7$ is

stable for at least six months in an all-glass vessel at room temperature.

Cadmium iodide-starch reagent. In order to estimate micro-quantities of organic material it is necessary to use very dilute solutions of $\text{K}_2\text{Cr}_2\text{O}_7$ for the oxidation. Hence a sensitive method is required for determination of the excess of $\text{K}_2\text{Cr}_2\text{O}_7$. The use of unstable KI and starch solutions was avoided by employing one solution, the CdI_2 -starch reagent of Lambert (1951). This reagent is colourless, highly sensitive and stable for at least a month when prepared with soluble starch as described under 'Analytical Procedure'. Lambert & Rhoads (1956) found that the reagent was even more stable (for 6 months) when pure linear starch fraction ('amylose') was used. They prepared this fraction by a method which uses a combination of the procedures of Krishnaswamy & Sreenivasan (1948)

Fig. 2. Effect of substrate 'concentration' on the breakdown of insoluble cellulose to soluble products by enzyme preparations from *Myrothecium verrucaria*. A volume (0.5 ml.) of standard enzyme preparation in Sørensen phosphate buffer (pH 5.5, 0.067 M- Na_2HPO_4 + 0.067 M- KH_2PO_4) was incubated at 37° for 1 hr. with the substrate, swollen cellulose powder (Whatman) (Halliwell, 1957). x, Loss of weight of insoluble cellulose determined from the residual weight of cellulose after incubation with enzyme, followed by filtration and washing of cellulose (Halliwell, 1958); O, soluble saccharides produced (estimated as glucose, expressed as cellulose). The soluble saccharides were separated by filtration and determined by iodometric estimation of excess of $\text{K}_2\text{Cr}_2\text{O}_7$. The protein of the enzyme used gave a negligible dichromate value in comparison with that of the soluble saccharides formed.

Table 3. *Recovery of cellobiose, sucrose and starch as measured by the iodometric micro-procedure*

Approximately 10–40 $\mu\text{g.}$ of the carbohydrates was estimated by the iodometric micro-procedure and the results were calculated as anhydroglucose from a standard glucose calibration curve.

Carbohydrate	Amount taken in 0.45 ml. of soln. ($\mu\text{g.}$)	Amount found (mean value, %)	Range of recovery (%)
Cellobiose	41	101	99–103
	8.6	99	97–101
Sucrose	41	101	101–102
	8.9	97	96–100
Starch	32	98	97–100
	9.1	96	95–99

and of Schoch (1945). We have obtained the linear starch fraction by the same methods and used it to prepare the CdI_2 -starch reagent when it gave readings 5–10% greater than did soluble starch. Use of the purified starch, however, was discontinued when it was found that CdI_2 -starch reagent prepared with commercially-available soluble starch was stable for at least a month. The stability of the reagent was examined by adding 1 ml. of the solution to 0.5 ml. of 0.003% (w/v) aqueous $\text{K}_2\text{Cr}_2\text{O}_7$ in 2.2 ml. of 1.63N- H_2SO_4 in a total volume of 10 ml. with water. The blue colour was developed for 20 min. and read as described above.

Application of the method to the determination of cellulose breakdown by enzymic preparations. The iodometric micro-procedure has been used to determine the formation of soluble carbohydrates formed from different amounts of cellulose by enzymes in a culture filtrate from *Myrothecium verrucaria* (Fig. 2).

The soluble products were separated by filtration through sintered glass (porosity 3) with or without prior centrifuging. Centrifuging by itself is insufficient to precipitate the finer particles of cellulose which remain floating on the supernatant fluid, a difficulty encountered previously (Halliwell, 1958). In addition to determining soluble products we also determined residual cellulose as described earlier (Halliwell, 1958) in order to obtain a direct estimate of the amount of insoluble cellulose which had been made soluble. The experiment in Fig. 2 was designed to study the effect of the 'concentration' of insoluble cellulose on the amount of insoluble cellulose made soluble by a fixed amount of enzyme. There is reasonable agreement between the results obtained by the two methods used for the determination of cellulolysis. The enzyme appears to be saturated with insoluble cellulose at about 20 mg.

DISCUSSION

The main errors of the iodometric micro-method were found to be associated with delivery of the dilute solution of $\text{K}_2\text{Cr}_2\text{O}_7$ in 97% H_2SO_4 . This was largely overcome by the use of a pipette with a drawn-out tip similar to that of a Warburg pipette (Gallenkamp, cat. no. a 12229). If necessary the tip of the pipette may be ground down to give an orifice sufficiently wide (approximately 1 mm.) to allow a reasonable rate of outflow of the $\text{K}_2\text{Cr}_2\text{O}_7$ - H_2SO_4 mixture. The pipette tip is immersed in the reagent to the minimum depth necessary for filling, and excess of solution is wiped off with glass-fibre papers (Whatman).

The reaction of $\text{K}_2\text{Cr}_2\text{O}_7$ - H_2SO_4 is rapid when carbohydrates are oxidized (7 min. heating period) and temperatures between 95° and 100° are equally satisfactory. Proteins require 20 min. heating and

it is important to maintain the water bath at boiling point.

We have used the iodometric-dichromate procedure to determine the total soluble material produced by enzymic degradation of insoluble cellulose. In order to examine true enzymic breakdown of insoluble cellulose it is necessary to determine residual insoluble cellulose or alternatively to determine the total soluble carbohydrates produced by the assay medium in the presence and absence of enzyme (Halliwell, 1959). An important feature of the present method when used to follow cellulolysis, in contrast with those methods dependent on the measurement of the formation of 'reducing' sugars, is that it enables one to measure and correct for that fraction of the substrate which consists of small molecules, whether reducing or non-reducing, that are soluble in the assay medium in the absence of enzyme. This fraction, we have found, can on occasions amount to 1–2% of the cellulose added and could presumably be attacked by β -glucanases (Festenstien, 1959) in the enzyme preparation. It is perhaps significant that most of the 'cellulases' in the literature will accomplish no more than this degree of dissolution of insoluble substrate. This fraction cannot be corrected for in the usual reducing-sugar methods, since the soluble small-chain polysaccharides have negligible reducing power. In the 'weight-loss' method for following cellulolysis (Halliwell, 1958), the soluble fraction is automatically corrected for, since the degree of cellulolysis is calculated from the substrate control, taken as 100% initial cellulose.

SUMMARY

1. An iodometric micro-method is described for the quantitative determination of carbohydrates and proteins by oxidation with a solution of potassium dichromate in concentrated sulphuric acid. The excess of reagent is determined colorimetrically by the addition of a starch-iodide solution. Glucose, cellobiose, sucrose and starch can be determined from a calibration curve for glucose.

2. The method covers the range 9–40 μg . of carbohydrates and proteins. Recovery for cellobiose at about 9 μg . is 99% (range 97–101%), and at 41 μg . 101% (range 99–103%); for soluble starch at 9 μg . recovery is 96% (range 95–99%) and at 32 μg . 98% (range 97–100%).

3. The procedure is recommended for the determination of micro-quantities of carbohydrates, proteins and their breakdown products formed enzymically or otherwise, for following purity of enzymes or proteins during fractionation procedures and for analysis of chromatographic eluates.

4. The procedure has been applied to determine the conditions which control the breakdown of

insoluble cellulose to soluble products by enzymes from *Myrothecium verrucaria*.

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The Effect of Hypophysectomy of the Rat and of Treatment with Growth Hormone on the Incorporation *in vivo* of Radioactive Amino Acids into the Proteins of Subcellular Fractions of Rat Liver

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The liver of the rat, like the body as a whole, decreases in size and protein content as a result of hypophysectomy, and this fall can be prevented to a large extent by treatment of the rat with growth hormone (Simpson, Evans & Li, 1949). There is some evidence that growth hormone stimulates growth by increasing the rate of protein biosynthesis (Russell, 1955) rather than by slowing down the rate of catabolism of proteins.

Zamecnik & Keller (1954) have described the preparation of a cell-free system from rat liver which brings about the incorporation of radioactive amino acids into protein *in vitro*. In the soluble fraction of the cell the amino acids are prepared for incorporation into proteins by amino acid-activating enzymes (Hoagland, Keller & Zamecnik, 1956) and by forming an amino acid-soluble ribonucleic acid intermediate (Hoagland, Zamecnik & Stephenson, 1957). In this system the assembly of the polypeptide chains occurs in the ribonucleo-protein microsomal particles of the endoplasmic reticulum (Littlefield, Keller, Gross & Zamecnik, 1955).

With a system of the type described by Zamecnik & Keller (1954), Korner (1958, 1959) has shown that the microsomes from the livers of hypophysectomized rats are significantly less able than those from livers of normal rats to assemble amino acids into proteins. Hypophysectomy of the rat did not result in any change, that was detectable by the system used *in vitro*, in the ability of the soluble fraction of the liver cells to prepare amino acids for incorporation. Treatment of hypophysectomized rats with growth hormone restored, to some extent,

the diminished ability of the liver microsomes to incorporate amino acids into protein. Treatment of normal rats with growth hormone enhanced the ability of liver microsomes to incorporate amino acids into protein.

The experiments described in this paper were carried out to see how far these findings *in vitro* could be confirmed and extended by experiments on the incorporation of amino acids into proteins in whole animals.

METHODS

Animals. Young, adult, female albino rats, of the same age and body weight, were selected from an inbred strain and were placed, at random, into two groups. The rats of one group were hypophysectomized under ether anaesthesia by the parapharyngeal approach (Ingle & Griffith, 1942), and the rats of the second group were submitted to a sham operation. After recovery from the operation each rat was housed in an individual cage and all rats were given the same ration of powdered diet (diet 41, Bruce & Parkes, 1949) made into a thick paste with water. The daily ration, established by trial and error, was the largest quantity of food which was entirely consumed by every rat (approx. 10 g./100 g. of rat/day). Water was freely available. The animals were kept on this ration for 3 weeks before being used in an experiment. The rats were not starved during the night before they were killed although each rat had eaten its last ration several hours before it was killed.

Growth hormone. Ox growth hormone, prepared by the method of Wilhelmi, Fishman & Russell (1948), was used. It was dissolved in 0.9% NaCl which had been adjusted to pH 7.5–8.0 with 0.1N-NaOH. The dose was injected subcutaneously in 0.5 ml. of 0.9% NaCl and the control rats were given 0.5 ml. of 0.9% NaCl by subcutaneous injection.

Radioactive compounds. Generally (G) labelled L-[G-¹⁴C]-leucine, DL-[1-¹⁴C]leucine and hydrolysed algal protein