cent V/V) R. Add *0.1 M sodium hydroxide* until a violet-blue colour is obtained, without exceeding the end-point. Place the solution in the test-tube (*D*). Without interrupting the stream of carbon dioxide, remove the funnel (*B*) and introduce through the opening into the flask (*A*) 25.0 g of the substance to be examined (*m* g) with the aid of 100 ml of *water R*. Add through the funnel 80 ml of *dilute hydrochloric acid R* and boil for 1 h. Open the tap of the funnel and stop the flow of carbon dioxide and also the heating and the cooling water. Transfer the contents of the test-tube with the aid of a little *water R* to a 200 ml wide-necked, conical flask. Heat on a water-bath for 15 min and allow to cool. Add 0.1 ml of a 1 g/l solution of *bromophenol blue R* in *alcohol (20 per cent V/V) R* and titrate with *0.1 M sodium hydroxide* until the colour changes from yellow to violet-blue (V_1 ml). Carry out a blank titration (V_2 ml).

Calculate the content of sulphur dioxide in parts per million from the expression:

$$32\,030 \times (V_1 - V_2) \times \frac{n}{m}$$

n = molarity of the sodium hydroxide solution used as titrant.

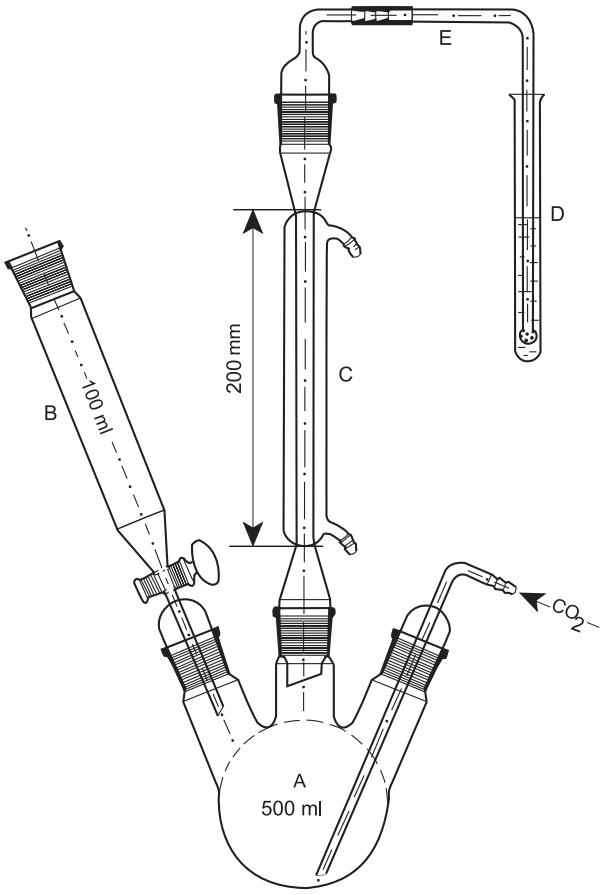

Figure 2.5.29.-1.– *Apparatus for the determination of sulphur dioxide*

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2.5.30. OXIDISING SUBSTANCES

Transfer 4.0 g to a glass-stoppered, 125 ml conical flask and add 50.0 ml of *water R*. Insert the stopper and swirl for 5 min. Transfer to a glass-stoppered 50 ml centrifuge tube and centrifuge. Transfer 30.0 ml of the clear supernatant liquid to a glass-stoppered 125 ml conical flask. Add 1 ml of *glacial acetic acid R* and 0.5 g to 1.0 g of *potassium iodide R*. Insert the stopper, swirl, and allow to stand for 25 min to 30 min in the dark. Add 1 ml of *starch solution R* and titrate with *0.002 M sodium thiosulphate* until the starch-iodine colour disappears. Carry out a blank determination. Not more than 1.4 ml of *0.002 M sodium thiosulphate* is required (0.002 per cent, calculated as H_2O_2).

1 ml of *0.002 M sodium thiosulphate* is equivalent to 34 µg of oxidising substances, calculated as hydrogen peroxide.

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2.5.31. RIBOSE IN POLYSACCHARIDE VACCINES

Test solution. Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 2.5 µg to 25 µg of ribose. Introduce 0.20 ml and 0.40 ml of the diluted solution into tubes in triplicate.

Reference solutions. Dissolve 25 mg of *ribose R* in *water R* and dilute to 100.0 ml with the same solvent (stock solution containing 0.25 g/l of ribose). Immediately before use, dilute 1 ml of the stock solution to 10.0 ml with *water R* (working dilution: 25 mg/l of ribose). Introduce 0.10 ml, 0.20 ml, 0.40 ml, 0.60 ml, 0.80 ml and 1.0 ml of the working dilution into 6 tubes.

Prepare a blank using 2 ml of *water R*.

Make up the volume in each tube to 2 ml with *water R*. Shake. Add 2 ml of a 0.5 g/l solution of *ferric chloride R* in *hydrochloric acid R* to each tube. Shake. Add 0.2 ml of a 100 g/l solution of *orcinol R* in *ethanol R*. Place the tubes in a water-bath for 20 min. Cool in iced water. Measure the absorbance (*2.2.25*) of each solution at 670 nm using the blank as the compensation liquid. Draw a calibration curve from the absorbance readings for the 6 reference solutions and the corresponding content of ribose and read from the curve the quantity of ribose in the test solution for each volume tested. Calculate the mean of the 3 values.

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2.5.32. WATER: MICRO DETERMINATION

PRINCIPLE

The coulometric titration of water is based upon the quantitative reaction of water with sulphur dioxide and iodine in an anhydrous medium in the presence of a base with sufficient buffering capacity. In contrast to the volumetric method described under (*2.5.12*), iodine is produced electrochemically in the reaction cell by oxidation of iodide. The iodine produced at the anode reacts immediately with the water and the sulphur dioxide contained in the reaction cell. The amount of water in the substance is directly proportional to the quantity of electricity up until the titration end-point. When all of the water in the cell has been consumed, the end-point is reached and thus an excess of iodine appears. 1 mole of iodine corresponds to 1 mole of water, a quantity of electricity of 10.71 C corresponds to 1 mg of water.

Moisture is eliminated from the system by pre-electrolysis. Individual determinations can be carried out successively in the same reagent solution, under the following conditions:

– each component of the test mixture is compatible with the other components,
– no other reactions take place,
– the volume and the water capacity of the electrolyte reagent are sufficient.

Coulometric titration is restricted to the quantitative determination of small amounts of water, a range of 10 µg up to 10 mg of water is recommended.

Accuracy and precision of the method are predominantly governed by the extent to which atmospheric moisture is excluded from the system. Control of the system must be monitored by measuring the amount of baseline drift.

APPARATUS

The apparatus consists of a reaction cell, electrodes and magnetic stirrer. The reaction cell consists of a large anode compartment and a smaller cathode compartment. Depending on the design of the electrode, both compartments can be separated by a diaphragm. Each compartment contains a platinum electrode. Liquid or solubilised samples are introduced through a septum, using a syringe. Alternatively, an evaporation technique may be used in which the sample is heated in a tube (oven) and the water is evaporated and carried into the cell by means of a stream of dry inert gas. The introduction of solid samples into the cell should in general be avoided. However, if it has to be done it is effected through a sealable port; appropriate precautions must be taken to avoid the introduction of moisture from air, such as working in a glove box in an atmosphere of dry inert gas. The analytical procedure is controlled by a suitable electronic device, which also displays the results.

METHOD

Fill the compartments of the reaction cell with *electrolyte reagent for the micro determination of water R* according to the manufacturer's instructions and perform the coulometric titration to a stable end-point. Introduce the prescribed amount of the substance to be examined into the reaction cell, stir for 30 s, if not otherwise indicated in the monograph, and titrate again to a stable end-point. In case an oven is used, the prescribed sample amount is introduced into the tube and heated. After evaporation of the water from the sample into the titration cell, the titration is started. Read the value from the instrument's output and calculate if necessary the percentage or amount of water that is present in the substance. When appropriate to the type of sample and the sample preparation, perform a blank titration.

VERIFICATION OF THE ACCURACY

Between two successive sample titrations, introduce an accurately weighed amount of water in the same order of magnitude as the amount of water in the sample, either as *water R* or in the form of *standard solution for the micro determination of water R*, and perform the coulometric titration. The recovery rate is within the range from 97.5 per cent to 102.5 per cent for an addition of 1000 µg of H_2O and in the range from 90.0 per cent to 110.0 per cent for the addition of 100 µg of H_2O.

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corrected 6.0

2.5.33. TOTAL PROTEIN

Many of the assay methods described in this chapter can be performed using kits from commercial sources.

METHOD 1

Protein in solution absorbs ultraviolet light at a wavelength of 280 nm, due to the presence of aromatic amino acids, mainly tyrosine and tryptophan, in the protein structure. This property can be used for assay purposes. If the buffer used to dissolve the protein has a high absorbance relative to that of water, an interfering substance is present. This interference may be obviated by using the buffer as compensation liquid but if the interfering substance produces a high absorbance, the results may nevertheless be compromised. At low concentrations, protein adsorbed onto the cell may significantly reduce the content in solution. This can be prevented by preparing samples at higher concentration or by using a non-ionic detergent in the preparation.

Test solution. Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a protein concentration between 0.2 mg/ml and 2 mg/ml.

Reference solution. Prepare a solution of a suitable reference substance for the protein to be determined, in the same buffer and at the same protein concentration as the test solution.

Procedure. Keep the test solution, the reference solution and the compensation liquid at the same temperature during the performance of this test. Determine the absorbances (*2.2.25*) of the test solution and the reference solution in quartz cells at 280 nm, using the prescribed buffer as the compensation liquid. The response must be linear in the range of protein concentrations to be assayed to obtain accurate results.

Light scattering. The accuracy of the determination of protein can be diminished by the scattering of light by the test sample. If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250 nm to 300 nm), scattering of the light beam results in an apparent increase in absorbance of the test sample. To calculate the absorbance at 280 nm due to light scattering, determine the absorbances of the test solution at wavelengths of 320 nm, 325 nm, 330 nm, 335 nm, 340 nm, 345 nm and 350 nm. Plot the logarithm of the observed absorbance against the logarithm of the wavelength and determine the standard curve best fitting the plotted points by linear regression. Extrapolate the curve to determine the logarithm of the absorbance at 280 nm. The antilogarithm of this value is the absorbance attributed to light scattering. Correct the observed values by subtracting the absorbance attributed to light scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a 0.2 µm filter that does not adsorb protein or clarification by centrifugation may be performed to reduce the effect of light scattering, especially if the solution is noticeably turbid.

Calculations. Use corrected values for the calculations. Calculate the concentration of protein in the test solution (C_U) from the following equation:

$$C_U = C_S \, (A_U / A_S)$$

where C_S is the concentration of protein in the reference solution and A_U and A_S are the corrected absorbances of the test solution and the reference solution, respectively.

METHOD 2

This method (commonly referred to as the Lowry assay) is based on the reduction by protein of the phosphomolybdotungstic mixed acid chromogen in the phosphomolybdotungstic reagent, which results in an absorbance maximum at 750 nm. The phosphomolybdotungstic reagent reacts primarily with