Analytical methods for bacterial fermentations

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ANALYTICAL METHODS

FOR

BACTERIAL FERMENTATIONS

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BY

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INTRODUCTION

The methods now being used in this laboratory, for the study of bacterial fermentations are described in detail in the following pages. This report is a revision of a report bearing the same title which was first issued in August, 1950. The 1950 report was an extension and a revision of a report entitled "Analytical Methods for the Bacillus subtilis Fermentation" which was printed in June, 1946. Requests for copies of these reports have exhausted the supply. In order to fill future requests this report was written to replace the previous ones with more up-to-date material. Actually some of the procedures in the first report, which were not included in the second one, have been resurrected since they are quite useful in certain instances. A few new methods have been added, mainly those dealing with the fermentation of carbon-14 labelled substrates.

The methods have been used chiefly in studies on fermentation of glucose by yeast and various species of bacteria producing 2,3-butanediol. Some of the procedures are original and some are modifications of well known procedures, while others have been adopted with little or no change. All of them have been tested on complex mixtures containing compounds likely to be found in fermentation solutions. These methods are collected together in order to have a laboratory "handbook" for the analysis of solutions fermented by the 2,3-butanediol bacteria. These methods should also be applicable to many other bacterial fermentations, but this collection of procedures cannot be considered complete since certain compounds formed under aerobic conditions, such as gluconic and keto-gluconic acids, are not dealt with.

Some of the changes introduced during the past six years have been due to a need for rapid methods suitable for screening a large number of bacterial cultures. Colorimetric methods are used in this type of analysis when possible because of their inherent rapidity when large numbers of solutions are to be analysed. Some micro-diffusion methods have been used for the same reason. Other changes have been made in order to increase the specificity of the methods. Partition chromatography has been found to be quite a useful technique for this purpose, making it possible to separate members of a homologous series of organic compounds on a small scale. Some procedures useful in tracer experiments involving carbon-14 have been added since the last revision.

PART I. VOLUMETRIC MEASUREMENT OF LIQUIDS.

Much of the time spent in the analysis of fermentation solutions is consumed in measuring volumes of liquid accurately,

either in titrating or pipetting solutions or making up known dilutions. This is true of both colorimetric and volumetric methods of analysis. It is important that the techniques used be as rapid and convenient as is possible for the degree of accuracy needed. Errors greater than one per cent should not be introduced by the volumetric measurements in the methods described in this report.

Syringes have several advantages over burettes and pipettes as devices for measuring volumes of liquids. The amount delivered by a syringe is not affected much by the speed of delivery or the viscosity of the liquid since there is virtually no drainage error. Viscous liquids such as sulfuric acid or glycerol can be rapidly and accurately delivered by a syringe.

The micrometer syringe, first described by Trevan (Biochem. J. 19: 1111 (1925)), makes a very useful general purpose burette if a 10 ml. syringe is used. Nearly all titrations in this laboratory are performed using a micrometer syringe such as that shown in Figure 1. This design (due to M.D. Chisholm of these laboratories) differs from previously published ones in a few small but important details which make the instrument easy to assemble or take apart for cleaning. A 10 ml. glass syringe (Becton Dickinson 10Y) is permanently mounted in rubber on an aluminium rod. A micrometer screw (Welch Scientific Co. 179E) is mounted in alignment with it on the same rod. The micrometer screw mounting can be quickly removed after loosening it by a quarter-turn of the thumb screw. The syringe plunger is then easily removed for cleaning. After the plunger has been replaced the instrument can be readily assembled since the thumb screw fits in a slot which automatically aligns the micrometer with the syringe. The removable glass tip is made by drawing out a Becton Dickinson 191P glass adapter. The micrometer syringe is clamped to a burette stand in a vertical position by the aluminum mounting rod, using an ordinary burette clamp. Collars of 26 gauge chromel wire with two projecting ends bent in the form of hooks are used to fasten the rubber bands to the syringe plunger and glass tip as shown in Figure 1. The micrometer screw has 2500 divisions and will eject about 4 ml. of liquid over this range. This means that 10 samples can be titrated, using about 0.4 ml. of reagent for each, with one burette filling.

The micrometer syringe is calibrated by ejecting five equal portions of water over the whole graduated range and weighing each in a 25 ml. glass stoppered Erlenmeyer. The syringes tested so far have been quite uniform, not more than 0.5% difference being found between any two portions. An average factor, called the micrometer factor in the following pages, is calculated from the results. It is used to convert micrometer units to ml. of liquid. Usually one micrometer unit equals about .00165 ml., but this varies with different syringes and each one must be calibrated.

The syringe should run smoothly when the micrometer screw

is turned. If it is mounted too tightly or out of alignment it will shudder and deliveries will not be reproducible. The point of the micrometer screw is rounded so it will rotate easily on the head of the plunger; the plunger itself should not turn.

When the syringe is filled the first time for a set of titrations, it is partly filled, inverted and all air ejected. It is then clamped in position for titration and may be completely filled by immersing the glass tip in the reagent and turning back the micrometer screw. The joint between the glass tip and the syringe can usually be lubricated well enough by the reagent being used, but it may be necessary at times to use a light application of vaseline.

Use of the micrometer syringe in place of ordinary burettes has a number of important advantages. It is free from errors due to drainage or faulty stop-cocks. About 5-10 times as many samples can be titrated on one filling due to the larger number of divisions. They are more easily cleaned. Small amounts of strong reagents are used in place of larger volumes of weaker ones, thus saving storage space for solutions, avoiding the instability often associated with weak solutions, and giving sharper endpoints. The solutions used are never weaker then 0.05 Normal and are usually in the range Normal to 0.1 Normal.

One ml. tuberculin syringes can be used to make a burette for accurately measuring one-tenth as much liquid as the above syringe. However, this scale is smaller than is needed for fermentation work and the 10 ml. micrometer syringe has been found more useful. The one ml. micrometer syringe is good for applying accurately measured amounts to paper chromatograms.

Syringes used free-hand make useful pipetting devices but maximum speed and accuracy are obtained by fitting them with mechanical stops. The design devised by Chaney (Ind. Eng. Chem.) Anal. Ed. 10: 326 (1938)) has been followed in this laboratory because it allows the greatest ease of cleaning of the syringes. The syringe shown in Figure 2 can be used to make rapid and accurate deliveries of 1, 2, 3, 4, or 5 ml. of a liquid. It is usually used without a needle. By pumping the liquid up and down several times air bubbles can usually be expelled without inverting the syringe. The liquid is then drawn in until the proper stud is above the ground flange of the syringe, then the plunger is rotated slightly and the excess liquid expelled as the stud is brought to rest on the flange. The syringe now contains the amount of liquid for which the stud is set and this may be ejected to obtain a reproducible amount of liquid with precision better than 0.5%. It is obvious that going from the 5 ml. stud to the 2 ml. stud will deliver 3 ml. of liquid, while going from the 5 ml. stud to the 1 ml. stud will deliver 4 ml. tThe studs are set by trial and error to give the required amounts of liquid on delivery, the instrument being calibrated accurately by weighing water. A ten ml. syringe similarly fitted with 6, 8 and 10 ml. studs is useful as well

as a one ml. tuberculin syringe fitted with 0.2 and 0.5 ml. studs. Glass tips are used on the latter but the 5 and 10 ml. sizes are used without tips. They are most useful for dispensing the equal amounts of a liquid into a number of flasks or test-tubes. The liquid being dispensed usually has to be poured into a beaker if no glass tip is used. Only the smallest size (tuberculin syringe with tip) is convenient to use for pipetting samples from volumetric flasks.

Recently we have used Chaney pipettes of a simpler construction. The aluminum heads are replaced by Lucite ones which are cemented on by a suitable adhesive. These heads are provided with a single stud which is long enough to set at any desired position. The setting can be made in a few seconds by using the graduation on the syringe barrel. This is suitable for most methods since the blank usually corrects for any deviation of the setting from the true volume, thus it is only necessary to be sure that each flask in the series has received the same amount.

Chaney pipettes enable accurate dilutions of large numbers of solutions to be made fairly rapidly. A measured amount of water is syringed into each of a series of test tubes and accurately measured samples then pipetted in. Since, in fermentation work, the samples are usually rather dilute aqueous solutions, the final volume in the test tubes can be taken as the sum of the volumes added. Chaney pipettes like these can be purchased from the Microchemical Specialties Co., Berkeley, California.

There are some automatic bulb pipettes (Alfred Bicknell Associates, Cambridge, Mass.) now on the market which are very rapid in their action and accurate enough for many purposes. These are used in this laboratory quite extensively, for the measurement of aqueous solutions.

Conventional pipettes are used chiefly for pipetting samples stored in test tubes or volumetric flasks and they have been replaced, even for this purpose, by the bulb pipettes and the small size Chaney pipettes (with tip) when possible.

PART II. RAPID ANALYSIS OF FERMENTATION SOLUTIONS.

Rapid methods of analysis are necessary for screening of large numbers of bacterial isolates in order to see which ones warrant further study. The methods should be accurate to within 5%, if possible, and the speed such that one analyst can do at least forty determinations in one day.

The following directions apply to the analysis of solutions which originally contained 5% of carbohydrate. The dilutions should be changed accordingly if the initial carbohydrate concentration is greater or less than this.

The fermentation solution is diluted 5 times by addition of water. An 0.05 ml. sample of each diluted solution is pipetted into a 25 mm. round cuvette (for Coleman Model 6 spectrophotometer) containing 3.95 ml. of water. The water is added to the cuvettes by a Chaney pipette (see page 4) and the samples measured by a fine bore pipette controlled by a screw. The anthrone reagent is added and total carbohydrates determined, as glucose, following Procedure 14. The range extends up to 3% glucose in the original solution.

All solutions containing more than 1.5% glucose are discarded and the remainder diluted further by pipetting 2 ml. into a rubber-stoppered test tube containing 8 ml. of water. This gives a solution diluted 25 times altogether.

One ml. of this solution is used for the determination of glycerol (Procedure 17), one ml. for the determination of 2,3-butanediol (Procedure 18) and one ml. for the determination of ethanol (Procedure 27).

The 25 times diluted solution is diluted 100 times more by pipetting 1 ml. into a 100 ml. volumetric flask and making it to volume. Care must be exercised to see that this very dilute solution is not contaminated with saliva or perspiration. Acetoin is determined on 5 ml. of this solution by Procedure 19 and lactic acid on 0.5 to 1 ml. by Procedure 23.

All calculations are based on the original undiluted fermentation solution. If the total apparent 2,3-butanediol, determined by Procedure 18, is less than 0.2%, acetoin determinations are not made. Some organisms give such high concentrations of lactic acid that it may be necessary to dilute a portion of the 2500 times diluted solution with an equal volume of water before analysing for it.

A calculation of the amount of glucose accounted for by each of these products is made as follows:

% 2,3-Butanediol x 2.00	= %	glucose	accounted	for
% Acetoin x 2.05	= %	"	"	"
% Ethanol x 1.96	= %	"	"	"
% Glycerol x 1.46	= %	"	"	"
% Lactic acid x 1.00	= %	"	"	"

The sum of these values should equal the difference between % glucose at the beginning and end of the fermentation.

The above factors are based on the assumption that each product is formed by definite reactions as follows:

(1) $C_6H_{12}O_6 \longrightarrow CH_3.CHOH.CHOH.CH_3 + 2CO_2 + H_2$

(2) $C_6H_{12}O_6 \longrightarrow CH_3.CHOH.CO.CH_3 + 2CO_2 + 2H_2$

(3) $C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$

(4) $C_6H_{12}O_6 \longrightarrow 2CH_3.CHOH.COOH$

(5) $3C_6H_{12}O_6 + 2H_2O \longrightarrow 4CH_2OH.CHOH.CH_2OH + 2CH_3COOH + 2CO_2$

Since glycerol might be formed by other reactions, as in the B. Subtilis fermentation

(6) $3\ C_6H_{12}O_6 \longrightarrow 2CH_3.CHOH.CHOH.CH_3 + 2CH_2OH.CHOH.CH_2OH + 4CO_2$

the above calculations are only approximate. However, they are useful for obtaining an idea of about how much glucose has been converted to unknown compounds. If the glucose unaccounted for is appreciable (more than 1/4 of the total) a determination of the total organic matter in the solution is made on 2 ml. of the 25 times diluted solution by Procedure 24. The total meq. of dichromate reduced by one ml. of the original solution is corrected for the meq. reduced by the compounds known to be present in the fermented solution to find meq. reduced by unknown compounds.

% 2,3-Butanediol x 1.30 = meq. dichromate reduced per ml. by 2,3-butanediol
% Acetoin x 1.43 = meq. dichromate reduced per ml. by acetoin
% Ethanol x 0.98 = " " " " " by ethanol
% Glucose x 1.33 = " " " " " by glucose
% Glycerol x 1.52 = " " " " " by glycerol
% Lactic Acid x 0.53 = meq. " " " " by lactic acid.

The meq. of dichromate reduded per ml. by unknown compounds is reported. It is roughly equivalent to the % of unknown compounds. The value of this determination lies in the fact that carbon dioxide and acetic acid do not react, hence the unknown compounds must be something more interesting. This makes it especially useful for analysis of aerobic cultures. It could also be used to follow the fractionation of the culture in isolation of unexpected products.

Other methods simple enough to be used for screening cultures are the determination of diacetyl (Procedure 20), acetone (Procedure 30) and isopropanol (Procedure 31). When these rapid methods are used for screening tests they are best applied for the determination of major products. Methods involving distillation, ether extraction and partition chromatography are not rapid enough, hence the fermentation solutions must be analysed without any treatment other than dilution. If the solutions only contain a small amount of a product, e.g., acetoin, it would have to be analyzed without much dilution and large errors might be caused by interfering substances, while solutions containing high amounts of acetoin can be diluted to

such an extent there would be little or no interference from compounds such as proteins or amino acids.

PART III. MATERIAL BALANCES FOR BACTERIAL FERMENTATIONS.

(a) Fermentation with continuous removal of gaseous products.

In order to obtain a material balance for a fermentation it is necessary to determine the gases formed as well as the products in solution. It may also be desirable to keep an atmosphere of approximately constant composition in contact with the fermenting solution. Both of these objectives can be realized if a gas such as air or nitrogen is bubbled continuously through the solution during fermentation and the exit gas is passed through a suitable combustion and absorption train.

The train shown in Figure 3 is suitable for maintaining anaerobic conditions by a stream of nitrogen and determining the carbon dioxide and hydrogen formed during fermentation. Nitrogen gas, from a cylinder fitted with a pressure reducing valve, passes through a Vycor tube, packed with reduced copper and maintained at 500-600°C in the furnace B and then through a soda-lime tower C and a gas washing bottle D (containing Normal NaOH) into the fermentation flask F. The gas from F passes through the cold trap H (immersed in dry ice in the thermos flask I) and then in succession through a drying tube J packed with anhydrous magnesium perchlorate (Anhydrone), an absorption tube K packed with Ascarite or Caroxite, a Vycor tube containing wire-form copper oxide heated at 450-550°C in Furnace L, another drying tube M packed with Anhydrone and a bubbler N containing concentrated sulfuric acid. The gas is freed of volatile liquid products and water by H, J is a safety tube removing any traces of water, K absorbs the carbon dioxide but not the hydrogen, the hydrogen is converted to water in L by the reaction

$$H_2 + CuO \longrightarrow Cu + H_2O$$

and the water thus formed is absorbed in M. Carbon dioxide and hydrogen are determined by weighing K and M before and after the fermentations.

The nitrogen is freed of oxygen and carbon dioxide by B, C and D. The rate of flow of gas through the train is controlled by the stopcock E and measured by counting the bubbles passing through N. Sufficient nitrogen pressure is used so that a slow stream of bubbles is passing out of the side arm in the water-filled pressure regulator A. This is necessary since actually six fermentation flasks with their gas analysis trains are connected with one purifying train by using a manifold with six stopcocks in place of E. As long as nitrogen is issuing from A it is possible to vary the rate of flow of gas through any one of the six parallel fermentation flasks without affecting the

others. The temperature of the fermentations is controlled by the constant temperature water bath G. An air bath or constant temperature room may be used.

The absorption tube K is packed with a shallow layer of Anhydrone on top of the Ascarite or Caroxite to prevent loss of water if a tube should happen to be reversed after being in use for a time. When carbon dioxide is absorbed the reaction is:

$$2\ NaOH + CO_2 \longrightarrow Na_2CO_3 + H_2O$$

and some of this water may be lost if this precaution is not taken. Actually Nesbitt bulbs are usually used for K and M rather than U-tubes as shown in this schematic diagram.

The Vycor tube in B is about 30 mm. O.D. and is packed for about 2 ft. of its length with wire-form copper oxide. The oxide reduced to copper by passing a slow stream of hydrogen through it at 350-450°C. The tube can be heated very conveniently by a quartz "Glas-Col" column heater controlled by a Variac transformer. After the reduction is complete the excess hydrogen is flushed out with nitrogen, the temperature is raised to 500-600°C. and it is ready for use. When it becomes oxidized again it may be regenerated in the same way. The Vycor tube in L is about 13 mm. O.D. and is packed for about 6" of its length with wire-form CuO, maintained at 450-550°C. When this becomes reduced by the hydrogen from anaerobic fermentations it can be regenerated, between runs, by passing air through it at the same temperature.

If air or oxygen is to be used in place of nitrogen, the train is the same as in Figure 3, with the omission of B.

The fermentations are run in flasks similar to those shown in Figure 4A. Usually about 150 ml. of liquid medium is used and 1-3% of sterile calcium carbonate added as a buffer. It is necessary to know exactly how much carbonate is added. At the end of the fermentation any residual carbonate is decomposed by addition of hydrochloric acid and the carbon dioxide released measured with that formed during the fermentation.

The following directions are for fermentations on the most frequently used medium, i.e., one containing 5% glucose and 0.5% yeast extract. All openings of the flask (Figure 4A) are plugged with cotton wool except for the opening closed by the serum bottle cap. Fifty ml. of a 15% glucose solution is accurately measured into the flask and it is autoclaved at 20 lb. pressure for 15 mins. At the same time a salts suspension (containing 0.25% KH_2PO_4, K_2HPO_4 0.20%, $MgSO_4.7H_2O$ 0.10%, $FeSO_4$ 0.025%, $CaCl_2$ 0.05% and NaCl 0.10%) and a 1.0% solution of Difco yeast extract are sterilized in separate Erlenmeyers. The glass stopper of the fermentation flask is also autoclaved, wrapped in paper, as well as some stopcock grease in a small beaker containing a glass rod and plugged with cotton wool. The serum bottle cap is sterilized in a Petri plate. The calcium carbo-

nate (about 3 gm.) is sterilized in a 50 ml. Erlenmeyer flask plugged with wool, in a hot air oven at 160°C for 3 hr. or more. The flask is cooled in a desiccator and weighed. The calcium carbonate is then dumped into the fermentation flask containing the cool, sterile glucose solution using aseptic technique and the flask and plug are again weighed. The amount of calcium carbonate added is recorded to the nearest mgm. The cool sterile salts solution (30 ml.) and yeast extract solution (75 ml.) are then put into the fermentation flask aseptically. The contents of the flask are mixed, the cotton plug is replaced by the serum bottle cap, and the flask is connected to the gas train and swept out for one hour. Tubes K and M (Fig. 3) are weighed during this period. The fermentation flask is then inoculated by 5 to 10 ml. of a 24 hr. culture of the organism (usually grown in a medium containing 1% glucose and 0.5% yeast extract) and the cotton plug in the neck replaced by the glass stopper coated with the sterile grease. The rate of flow of the gas is regulated to 60 - 70 bubbles per minute (12-14 ml.) through the sulfuric acid bubbler.

The carbon dioxide absorption tube is weighed daily in order to find when the fermentation is finished. When carbon dioxide evolution has practically stopped, 15 ml. of 5 Normal HCl is added to the fermentation flask through the serum bottle cap by a syringe fitted with a 22 gauge stainless steel needle. The carbon dioxide evolved is swept out overnight and then tubes K and M again weighed.

The increase in weight of tube K is due to the carbon dioxide formed from the sugar plus that released from the carbonate by the action of fermentation acids and hydrochloric acid. Since the total carbonate added is known, the carbon dioxide equivalent to this is subtracted to obtain the carbon dioxide formed from the sugar. The increase in the weight of M is due to water equivalent to the hydrogen formed in the fermentation. It follows from this that

$$\frac{\text{Mgm. increase in wt. of K}}{44} - \frac{\text{mgm. } CaCO_3 \text{ added}}{100} = \text{millimoles of carbon dioxide formed from glucose.}$$

$$\frac{\text{Mgm. increase in wt. of M}}{18} = \text{Millimoles of hydrogen formed from glucose}$$

The amount of solution fermented is greatly in excess of that needed for the estimation of most of the products. It is run on this scale to get an accurate measurement of the hydrogen;

(b) Fermentations in closed vessels

Material balances can be readily obtained on fermentations run in closed vessels with a volume of gas relatively large compared to that of the liquid. This technique was developed

for studies on fixation of carbon dioxide labelled with carbon-14. In experiments of this type it is obviously necessary to maintain the fermentation gas in contact with the fermenting solution. However the method is recommended, because of its simplicity, for other studies as well.

The flask shown in figure 4B is suitable for fermentations of this type. Anaerobic fermentations are conducted as follows. The flask containing an accurately weighed amount of glucose (0.5 gm.) and five ml. of water is plugged with cotton and sterilized. The stopcock is first removed, wrapped in paper and sterilized separately, the neck of the flask also being wrapped in paper to protect the seat of the cock. The salts solution, yeast extract solution and a suspension of calcium carbonate are sterilized separately. When the solutions are cool they are mixed and added to the flask. Their strength is such that the flask now contains ten ml. of a medium with five percent glucose, one-half percent yeast extract, and about three percent calcium carbonate. Experience has shown that the carbonate can be pipetted as a suspension with only 1-2% error provided finely divided material is used and mixed thoroughly immediately before pipetting with a coarse-tipped pipette. The solution is inoculated with one ml. of a suspension of cells or spores. The cotton plug is now replaced by a sterile serum bottle cap as shown in figure 4B and the stopcock plug lubricated with sterile grease and inserted.

The gas in the flask is replaced by evacuating three times and filling with purified nitrogen, the pressure being finally set at about 0.8 atmospheres. The nitrogen used for this purpose may be purified by hot Cu, as above, or by Fieser's solution using amalgamated zinc as a reducing agent (L.J. Brady, Anal. Chem. 20: 1033 (1948)). If aerobic conditions are desired the flask may be filled with oxygen or the original air left in.

Once the desired gas phase has been obtained the stopcock is closed and the flask incubated at an appropriate constant temperature. During this time samples of gas or liquid can be removed for analysis by means of a syringe equipped with a stainless steel needle. The needle is inserted through the serum bottle cap; with proper care contamination and loss of gas can be avoided. Usually the fermentations are run a predetermined time and then analysed.

When the fermentation is done 1.5 ml. of 5N HCl is injected into the still sealed flask to decompose carbonates. After thirty minutes or more a sample of gas is taken and analysed in a suitable apparatus to determine the relative proportions of CO_2, O_2 and H_2 or any other gases that might be present. A simple method of sampling and analysis is described in Procedure 32. The flask is then connected to a bead tower (Figure 11) filled with 20 meq. of carbonate-free NaOH and the stopcock opened. A gentle suction is applied to

the top of the tower and the serum bottle cap pierced immediately by a 22 gauge steel needle to allow a current of air to sweep out the remainder of the carbon dioxide into the tower. The needle is connected, by rubber tubing, to an alkali filled bubbler and a soda-lime tower to free the incoming air of carbon dioxide. Sweeping is continued for 35-40 minutes at a rate of 3-4 bubbles per second, with occasional shaking. The alkali containing the fermentation CO_2, as sodium carbonate, is made to a volume of 100 ml.; aliquots are analysed by precipitation as $BaCO_3$, (Procedures 25 and 33) or by titration (Procedure 26). If carbon-14 is to be measured the samples are mounted as described in Procedure 33.

The total amount of carbon dioxide is the sum of that caught in the bead tower and that removed for analysis. Analysis of the gas sample gives the ratio of the other gases to CO_2 and since the total amount of it is known the total amount of all the other gases can easily be calculated without knowledge of either the total volume or the pressure of the gases at the time of sampling. The error due to solubility of gases in water is not great enough to cause trouble when the ratio of gas/liquid is around fifty, as it is here. It is, however, necessary to know the amount of oxygen present at the start if oxygen consumption is to be calculated. This necessitates measuring the pressure, at a known temperature, with a flask of known volume, at the start of the experiment.

The closed-vessel technique is quite useful for experiments where the pH is adjusted by periodic addition of NaOH. The calcium carbonate is replaced by about the same amount of magnesium ammonium phosphate, which doesn't have to be measured accurately since it doesn't contribute to the gases. This slightly soluble salt buffers by dissolving near pH 6. A suitable indicator such as bromocresol purple is incorporated into the medium, The color of the indicator is observed periodically and the pH adjusted when necessary by injection of .01-.03 ml. of N- 3N NaOH by means of a one-quarter ml. syringe fitted with a 24 gauge steel needle. This is easily carried out aseptically. This procedure has been found useful with concentrated suspensions of cells, which are capable of fermenting a five percent glucose medium in about five hours. Phenol red or cresol red may be used to maintain a higher pH. The injections are required at 10-15 minute intervals and the end of the fermentation is shown by cessation of acid formation.

(c) Determination of non-gaseous products

The following directions for the 150 ml. fermentations can be scaled down for 10 ml. fermentations where necessary. The acid residue in the fermentation flask is cautiously neutralized to the phenol red endpoint by 5 Normal NaOH added slowly from a screw-controlled pipette with a fine tip.

immersed in the solution. The cold trap is thawed and its contents transferred to the fermentation flask by means of a pipette using 20-25 ml. of wash water as effectively as possible. The mixture is then clarified by zinc hydroxide as outlined in Procedure 1 using thenml. of the zinc sulfate solution. The mixture is made to a definite volume in a 250 ml. volumetric flask. It is advisable to avoid unnecessary dilution because the analytical procedures using partition chromatography require compounds to be present in 0.1-1.0% concentration.

The cleared solution is analysed directly for residual carbohydrate by Procedure 14, for glycerol by Procedure 17, and for 2,3-butanediol by Procedure 18, If 2,3-butanediol or glycerol is found in appreciable amounts, they should also be estimated by Procedure 12. Small amounts of 2,3-butanediol can also be determined by Procedure 28, and reducing sugars by Procedure 15 or 16 if desired.

A 10 ml. portion of the cleared solution is distilled by Procedure 3 and a small aliquot of the distillate, suitably diluted, is analysed for acetoin (Procedure 19), diacetyl if acetoin is present (Procedure 20), acetone (Procedure 29) and total alcohols (Procedure 8).

If there is an appreciable amount of alcohol present, isopropanol is determined by Procedure 31 and the other individual alcohols are determined on an undiluted portion of the distillate (Procedure 13).

A 5 ml. portion is acidified and extracted with ether (Procedure 2), the total acids measured and if there is an appreciable amount, the individual acids are determined using Procedure 10.

It is rather difficult to get a decent measurement of the amount of glucose carbon assimilated by the cells, unless glucose is the only source of carbon, since it is rather uncertain how much of the total carbon in the cells comes from the yeast extract. The total carbon in any fraction can be determined accurately (Procedure 25). If a washed zinc hydroxide precipitate (see Procedure 1) is analysed, the carbon in the cells is measured together with that of the proteins and in the case of synthetic media with glucose as the only carbon source, this can be taken as the carbon assimilated. However, if appreciable amounts of supplementary nutrients, such as yeast extract, have been added, a large blank correction is necessary and it is difficult to find how much this blank value is changed during an actual fermentation. Consequently any estimation of the glucose carbon assimilated in complex media is only very approximate.

(d) Calculation of material balances

The results of the analysis are usually calculated on the basis of millimoles of product per 100 millimoles of glucose fermented. A table is drawn up on this basis and then carbon balances and oxidation-reduction (O/R) balances calculated from it.

To calculate the carbon balances the millimoles of any product are multiplied by the number of carbon atoms in the molecule to get millimoles of C_1. When this has been done for all of them the results are added up. Since glucose is a C_6 compound a total of 600 millimoles of C_1 should be obtained. Usually only about 92-96% of this is found if the carbon assimilated is not determined.

The O/R balance is the ratio of the number of equivalents of oxidation and reduction that have occurred during the fermentation. Since there must be an equal amount of oxidation and reduction in any chemical reaction this ratio should have a value of unity. The O/R balances are calculated by multiplying the millimoles of product by a factor expressing its degree of oxidation or reduction compared to glucose. The figures are taken as positive if the compound is more highly oxidized than glucose and negative if more reduced. The sum of the positive values is divided by the sum of the negative values to obtain the O/R index for the fermentation. The factors for some common products are as follows:

2,3-Butanediol	-6	Carbon dioxide	+4
Acetoin	-4	Formic acid	+2
Diacetyl	-2	Succinic acid	+2
Acetone	-4	Lactic acid	0
Ethanol	-4	Acetic acid	0
Butanol	-8	Glycerol	-2
Hydrogen	-2	O_2 consumed	-4

The factor can be easily calculated for any compound from the hydrogen and oxygen in its molecular formula. In glucose the ratio of hydrogen to oxygen is 2 to 1, as in water. Any compound with this same ratio is multiplied by zero and hence does not enter into the O/R balance. However for ethanol, C_2H_6O, there are 4 H atoms more than are required to get the 2:1 ratio, hence it is multiplied by -4. On the other hand carbon dioxide requires 4 H atoms to get this ratio so it is multiplied by +4. O/R balances cannot be calculated for fermentations run under aerobic conditions unless the amount of oxygen consumed is measured. When fermentations are run with continuous removal of the gases, oxygen consumption is not measured hence O/R balances for this type of fermentation can only be drawn up if they are run anaerobically. Oxygen consumption can be measured using the closed-vessel technique. One mole of O_2 consumed is equivalent to two moles of H_2 produced so it is multiplied by -4. The O/R value is usually between 1.01 and 1.06 since the cells, which are more reduced than glucose, are not taken into account.

The following table illustrates the calculation of a material balance for the anaerobic dissimilation of glucose by a strain of Serratia marcescens:

Product	mM of product per 100 mM of glucose fermented	mM of C_1	Meq. of	
			Oxidation (+)	Reduction (-)
2,3-Butanediol	51.45	205.80	-	308.70
Acetoin	0.81	3.24	-	3.24
Glycerol	4.54	13.62	-	9 9.08
Ethanol	42.24	84.48	-	168.96
Lactic acid	33.09	99.27	-	-
Formic acid	39.80	39.80	79.6	-
Acetic acid	nil	-	-	-
Succinic acid	3.41	13.64	6.82	-
Carbon dioxide	106.1	106.1	424.4	
Hydrogen	0.52	-	-	1.04
		595.95 or 94.5%	510.8	491.02

Ratio O/R = 1.04

PART IV. ANALYTICAL PROCEDURES

PROCEDURE 1. Clarification of Fermentation Solutions

Reference: M. Somogyi - J. Biol. Chem. 86: 655 (1930).

Principle:

It is usually necessary to remove proteins from fermentation solutions to prevent excessive frothing or emulsification in the distillation or extraction procedures used in analysis. This can be done by precipitating zinc hydroxide

in the solution and then centrifuging it out. The supernatant liquid is clear and can usually be distilled or subjected to continuous extraction by ether without difficulty.

The culture is adjusted to pH 7-8 and treated with zinc sulfate and then with an equivalent amount of sodium hydroxide.

$$ZnSO_4 + 2NaOH \longrightarrow Zn(OH)_2 + Na_2SO_4$$

Since the zinc hydroxide is only very slightly soluble, the net effect of this treatment is addition of sodium sulfate and removal of proteins and other substances which interfere in subsequent analyses. However, since zinc hydroxide will dissolve in excess alkali, forming sodium zincate, it is important to avoid adding an excess of NaOH.

$$Zn(OH)_2 + 2NaOH \longrightarrow Na_2ZnO_2 + H_2O$$

This treatment was originally developed for removal of reducing substances in blood which interfere in determination of glucose by copper reduction methods. It does not remove any of the common fermentation products.

Reagents: 54, 80, 82, 105.

Procedure:

If a sufficient amount of solution is available the best procedure is to add the zinc sulfate solution to the fermentation solution and then titrate with sodium hydroxide to pH 7.6 - 7.8 using a glass electrode. The zinc sulfate solution added should be about 1/10 to 1/20 the volume of the fermentation solution. The solution is made to a definite volume, centrifuged and the supernatant liquid decanted and stored in a refrigerator. When only small amounts of solution are available it is first adjusted to pH 7-8 using the phenol red indicator. The zinc sulfate is then added using 1/15 the volume of the fermentation solution. After thorough mixing Normal sodium hydroxide, equivalent to the zinc sulfate, is added with shaking and the mixture made to a definite volume. It is centrifuged and the supernatant liquid decanted and stored as above. This liquid, suitably diluted, can be used for all the procedures given in this report.

PROCEDURE 2. Continuous Ether Extractions

Principle:

In making a careful examination of a fermentation solution, it is often advisable to fractionate it by ether extraction. The neutral cleared solution may be extracted first. (If the solution has not been cleared, emulsification may make the extraction difficult, if not impossible). 2,3-

Butanediol can be extracted completely along with about 10% of the glycerol while sugars, sugar alcohols and the salts of organic acids remain in the aqueous phase. If the solution is acidified with a highly ionized acid, such as hydrochloric acid, the organic acids are changed to the undissociated form which is readily extracted by ether while the excess HCl remains in the aqueous phase. Thus it is possible to obtain a neutral ether extract, which contains all of the 2,3-butanediol but no organic acids and an acid extract which contains all of the formic, acetic, propionic, butyric, succinic and lactic acids. The residual aqueous solution is free of these compounds but contains all the sugars or sugar alcohols and mineral salts, and about 85% of the glycerol originally present.

Apparatus:

Some of the extractors which have been found useful are shown in Figure 5. There are numerous designs to be found in the literature. Some of these go to great lengths in devising means of increasing the contact time between the ether and aqueous phases. However, in our experience the most important factor is the amount of ether that passes through a given amount of the aqueous phase in a given time. Thus the extraction rate with a given apparatus is roughly proportional to the rate of reflux of the ether. Since the capacity of an ordinary laboratory condenser is the limiting factor, the safest and most economical way to obtain a rapid extraction is to use a small volume of the aqueous phase. Using a rate of reflux of ether of approximately 5 ml. per minute, the smallest extractor (Figure 5C) gives practically complete extraction of 2,3-butanediol in 2 1/2 hrs. while the extractors in Figures 5B and 5A require about 6 hrs. and 44 hrs. respectively. The 10 ml. extractor (Figure 5B) is convenient to handle and is the one used most frequently for analysis. The 5 ml. extractor (Figure 5C) is used when a more rapid extraction is desired and the 120 ml. extractor (Figure 5A) is used chiefly for concentration of substances prior to isolation for formation of crystalline derivatives.

Condensers of the cold finger type are good since nearly all of the ether drops back down the center and enters the dispersion tube. The dispersion tube contains 2-4 small holes at the bottom each about 0.1 mm. in diameter. These are made by drawing out a fine peak of glass, grinding off the end and then warming it carefully in a small flame until it closes to the right diameter. This is found by trial and error. The holes should be fine enough so the ether in the dispersion tube rises to 2-4 cm. from the top when the extractor is operating at full speed. The extractors can be run six at a time on the six-place electrical heaters sold for use with Soxhlet extractors. It is safer to run them in a water bath at 60-70°C, however, A satisfactory bath can be made by placing an immersion heater below a sheet of copper, both being under water. The flasks are supported on the copper sheet.

Reagents: 29, 34, 54, 82.

Procedure:

(1) For large extractors (Figure 5A)

The dispersion tube is removed and 100 ml. of a neutral cleared solution (Procedure L) put in the extractor. The dispersion tube is replaced and the extractor connected to a 250 ml. boiling flask containing 90-100 ml. of ether, and a little water (5-10 ml.). It is then connected to the condenser and the extraction run 54 hrs. with the ether refluxing in an almost steady trickle (7-9 ml. per min.). The boiling flask is then changed for another one containing 70 - 80 ml. of fresh ether; at the same time 5 ml. of 5 Normal hydrochloric acid is pipetted into the distribution tube. It mixes in automatically when the extractor is started. The extraction of the acids is run for 24 hrs. The first fraction contains the 2,3-butanediol and about 10% of the glycerol and the second fraction contains the fermentation acids. If water is added and the ether evaporated on a steam bath until the mixture just becomes monophasic, aqueous solutions of these substances can be obtained with no appreciable loss. These aqueous solutions can be used for further analysis.

(2) For small extractors (Figure 5B)

Ten ml. of the neutral cleared solution is pipetted into the empty extractor. The dispersion tube is then replaced and 0.5 ml. of 5 Normal hydrochloric acid pipetted into it. The extractor is then connected to a 25 ml. Erlenmeyer flask (with a S.T. joint) containing 2-3 ml. of water and sufficient ether for proper operation (about 12 ml.). The extraction is run at the rate of about 5 ml. per minute for 6 hrs. if it is desired to extract all the 2,3-butanediol but only 4 hrs. if just the organic acids are wanted. Alternatively the 2,3-butanediol can be extracted from the neutral solution first and then the acids, in a separate fraction, after acidification with hydrochloric acid. This is not usually done now since ether extraction is usually employed to get an acid fraction suitable for partition chromatography, in which case the presence of a small amount of 2,3-butanediol is not objectionable. The extract, containing the organic acids, is warmed on a steam bath until the ether phase just disappears. A drop of phenol red is then added and the total acids determined by titration with Normal NaOH from a micrometer syringe. The neutralized solution is then evaporated to dryness in a 100 ml. beaker and dried overnight in a desiccator. The residual salts are saved for determination of the individual acids.

The smallest size extractors (Figure 5C) are used in the same way except only 5 ml. of solution is extracted. It is necessary to be very careful not to get this solution on the walls of the extractor since it would then be washed down into the boiling flask.

PROCEDURE 3. Distillation of Neutral Volatile Products

Reference: A.F. Langlykke and W.H. Peterson - Ind.Eng. Chem. Anal. Ed. 9: 163 (1937).

Principle:

The cleared fermentation solution from Procedure No. 1 (pH 7.5-8) contains the organic acids as non-volatile salts. On distillation these will remain behind and only neutral substandes, volatile with steam, will distill over. Compounds such as acetone, diacetyl, ethanol, isopropanol and n-butanol pass over completely in the first 50% of the distillate but only about half of the acetoin. However, the proportion of the acetoin distilling bears a fixed ratio to the proportion of the water distilling, if there is no fractionation of the distillate. The solutions are distilled in an apparatus designed to have little or no reflux and the distillation factor for acetoin determined on standard solutions. In the procedure given below 53.5 ± 1% of the acetoin is found in the distillate.

Procedure:

Exactly 10 ml. of the cleared fermentation solution (see Procedure 1) is pipetted into a dry 50 ml. boiling flask. Two glass beads are added and the solution distilled using the apparatus shown in Figure 6; exactly 5 ml. of distillate is collected in a marked test tube. This is tightly stoppered and stored in a refrigerator. The time required for each distillation is about 10 mins.

The asbestos insulation is intended to prevent reflux. A hole about the size of a dime is punched in the asbestos on the wire gauze in order to obtain a hot spot in the center of the bottom of the boiling flask; this promotes even boiling. A fast stream of cold water is run through the condenser to ensure good recovery of acetone.

Calculations:

Since all of the ethanol, acetone, diacetyl, isopropanol and n-butanol are recovered in the distillate, their concentration will be exactly twice that in the original cleared solution. Only 53.5% of the acetoin is in the distillate so its concentration will be 1.07 times that in the cleared solution. These concentrations should be kept in mind when calculating the best size aliquot to use in the various procedures for estimating these products.

PROCEDURE 4. Distillation of Volatile Acids

References: W.H. Olmstead, W.M. Whitaker and C.W. Duden - J. Biol. Chem. 85: 109 (1929).
E.P. Clark and F. Hillig - J. Assoc. Official Agr. Chem. 21: 684 (1938).

Principle:

If a solution of the salts of a volatile organic acid is treated with a strong non-volatile acid, such as sulfuric acid, the weaker acid exists largely in the undissociated state and may be distilled from the solution. Steam distillation is necessary since none of the organic acids are highly volatile. The order of distillation of the simple fatty acids from aqueous solutions is butyric, propionic, acetic and formic, with butyric distilling the most rapidly. This is the reverse of the order of distillation of the anhydrous acids. Apparently the acids are hydrated in aqueous solutions. The rather high degree of hydration of formic acid makes it difficult to recover quantitatively by distillation. However, the rate of distillation can be increased by addition of magnesium sulfate to the solution.

Reagents: 43, 54, 82, 97.

Procedure:

The apparatus shown in Figure 7 has been found quite useful. An aliquot of the cleared fermentation solution containing about 0.5 - 1.0 meq. of volatile acids is pipetted into the distillation flask and sufficient water is added to give a volume of 25 ml. One ml. of 10 Normal sulfuric acid is then added and 17-18 gm. of magnesium sulfate crystals. The flask is connected to the apparatus and the distillation started. The flame is adjusted to keep the level at about 25 to 30 ml. The distillation is run until about 250 ml. have been collected or until all the volatile acids are over as determined by titrating several successive 50 ml. portions of the distillate. The distillate is titrated with Normal NaOH, using the micrometer syringe, to the phenol red endpoint. The total volatile acids are recorded and the neutralized distillate then evaporated to dryness on a steam bath transferring to a 100 ml. beaker near the end. The residual salts are dried in a desiccator overnight and saved for determination of the individual acids by partition chromatography (Procedure 11).

Calculations:

Titer x micrometer factor x Normality of NaOH = meq. total volatile acids.

PROCEDURE 5. Oxidation by Periodic Acid Followed by Measurement of the Excess Periodate.

References: G. Frederick Smith - Analytical Applications of Periodic Acid and Iodic Acid. 5th Edition (1950).

E.L. Jackson - Organic Reactions, Vol. II, pages 341-375 (Wiley, 1944).

Principle:

Periodic acid oxidizes compounds with keto or hydroxyl groups on adjacent atoms. This method of oxidation, discovered by Malaprade, gives stoichiometric results with simple compounds. The end products are iodic acid, acids and aldehydes. Some examples are:

(1) $CH_3.CHOH.CHOH.CH_3 + HIO_4 \longrightarrow 2CH_3.CHO + HIO_3 + H_2O.$

(2) $CH_3.CO.CHOH.CH_3 + HIO4 \longrightarrow CH_3.CHO + CH_3COOH + HIO_3 + H_2O.$

(3) $CH_3.CO.CO.CH_3 + HIO_4 \longrightarrow 2CH_3.COOH + HIO_3 + H_2O.$

(4) $CH_2OH.CHOH.CH_2OH + 2HIO_4 \longrightarrow 2CH_2O + HCOOH + 2HIO_3 + H_2O.$

These reactions go smoothly and quantitatively in dilute aqueous solutions at room temperature. Diols, such as 1,3-butanediol, which do not have adjacent hydroxyl groups, and simple alcohols are not oxidized; neither are α-hydroxy acids, such as lactic acid, under the conditions used.

When organic compounds are oxidized by periodate the products can be predicted by assuming that the bonds between the two adjacent oxygen-bearing carbons are opened and the free valences thus formed satisfied by addition of hydroxyl groups. A quantitative measurement of the organic compound can be obtained by using a measured amount of periodic acid and then determining the amount remaining at the end of the reaction. The amount of organic compound is then calculated on the basis of the periodate consumed, using the appropriate equation.

The excess periodate can be determined in the presence of iodate by the reaction:

$$IO_4^- + 2I^- + 2H^+ \longrightarrow IO_3^- + H_2O + I_2$$

It is important that the pH be above 5 or else iodate will also oxidize the iodide. For this reason the solution must be buffered. This can be accomplished quite simply by adding an excess of sodium bicarbonate. The iodine released must then be titrated by arsenite since the pH is too high for use of thiosulfate. The best results are obtained by

adding a measured excess of arsenite and then titrating with iodine. If the arsenite is added before the iodide there is never any appreciable amount of free iodine released into the medium. The iodide then serves to catalyse the reduction of periodate to iodate by arsenite.

Reagents: 40, 51, 63, 73, 75, 94, 99.

Procedure:

Two ml. of Normal sulfuric acid is pipetted into a 125 ml. Erlenmeyer flask and exactly one ml. of 0.1 Molar periodic acid added. The sample, containing up to 3.5 mgm. of glycerol or up to 7 mgm. of 2,3-butanediol, is then added and enough water to make a volume of approximately 10 ml. The contents of the flask are then mixed and allowed to stand 1 hr. at room temperature (it may stand longer if more convenient). Approximately 5 ml. of 6.8% sodium bicarbonate solution is then added and, after thorough mixing, exactly 2 ml. of the 0.11 Normal sodium arsenite solution. Two drops of 50% KI are mixed in and the flask allowed to stand 10 mins. or more, at room temperature. Four drops of the starch indicator are added, the solution titrated with 0.1 Normal iodine solution, to a definite blue color, using the micrometer syringe. A blank is run in parallel.

Calculation:

Titer of sample - titer of blank = titer equivalent to periodic acid reduced.

Therefore:

(Titer of sample - titer of blank) micrometer units x micrometer factor x Normality of iodine = Meq. periodic acid reduced.

One meq. of periodic acid is reduced by: 45 mgm. of 2,3-butanediol
44 mgm. of acetoin
43 mgm. of diacetyl
23 mgm. of glycerol

PROCEDURE 6. Determination of Acids Formed During Oxidation by Periodate

References: I.S. Shupe - J. Assoc. of Off. Agr. Chemists, 26: 249 (1943).

Brockmann, Manna and Stark - Report from J.E. Seagram and Sons, Louisville, Ky.

Principle:

The test solution is treated with excess sodium

periodate after being adjusted to the methyl red endpoint. This oxidizes compounds such as glycerol, acetoin, diacetyl and mannitol to carboxylic acids, stoichiometrically (see Procedure 5 for reactions). The increase in the acidity is measured, after the oxidation is complete, by titration to the phenolphthalein endpoint. Since periodic acid titrated as a dibasic acid and iodic acid does not it is necessary to treat the solution with excess 2,3-butanediol, to convert all periodate to iodate before titrating to phenolphthalein if accurate values are to be obtained. This gives the sum of the acetic and formic acids formed during oxidation by periodate. A blank is run; it should only be 0.002 - .004 meq. This procedure does not oxidize glucose quantitatively but only to ca 70% of the theoretical value.

Reagents: 15, 36, 45, 52, 82, 86.

Procedure:

A 2-25 ml. aliquot of the test solution is made to about 40 ml. with water and adjusted to the methyl red end point with dilute HCl and N NaOH. The final adjustment is made by adding the N NaOH from the micrometer syringe; 10 ml. of the $NaIO_4$ solution is then added, the solutions mixed and allowed to stand 1-3 hrs. at room temperature. A blank is run. At the end of oxidation 5 ml. of the 10% 2,3-butanediol solution is added. After 10 mins. more a few drops of phenolphthalein is added and the solution titrated to the phenolphthalein end point with Normal NaOH using the micrometer syringe. The methyl red does not interfere as the solution turns yellow between the methyl red and phenolphthalein end points. The titers are corrected for the small blank values. It is important that an excess of periodate be used for the oxidation; a 10% excess is quite sufficient.

Calculation:

Micrometer units (corrected for blank) x micrometer factor x normality of base = meq. of acid formed during periodate oxidation. One mM of acetoin or of glycerol gives one meq. of acid.

PROCEDURE 7. Oxidation by Alkaline Iodine followed by Measurement of the Excess Iodine

References: L.F. Goodwin - J. Am. Chem. Soc. 42: 39 (1920).
C.L. Hinton and T. Macara - Analyst 49: 2 (1924).
A.F. Langlykke and W.H. Peterson - Ind. Eng. Chem. Anal. Ed. 9: 163 (1937).

Principles:

Alkaline iodine solutions will oxidize certain compounds

such as glucose, acetone and acetoin stoichiometrically. The amount of iodine consumed is measured after acidification, by titration with sodium thiosulfate.

An aqueous solution of the substance in question is treated with alkali followed by iodine. The iodine is converted to NaOI.

(1) $I_2 + 2NaOH \longrightarrow NaOI + NaI + H_2O$.

The hypoiodite oxidizes the organic compounds, rapidly as follows:

(2) $CH_3.CO.CH_3 + 3NaOI \longrightarrow CHI_3 + CH_3COONa + 2NaOH$

(3) $CH_3.CO.CHOH.CH_3 + +3NaOI \longrightarrow CHI_3 + CH_3CHOHCOONa + 2NaOH$

(4) $CH_2OH(CHOH)_4.CHO + NaOI + NaOH \longrightarrow CH2OH.(CHOH)_4.COONa + NaI + H_2O$

The excess NaOI is unstable, changing in a few minutes to a mixture of NaI and $NaIO_3$. For this reason it is important that the reagents be added in the proper order, for if the alkali and iodine are mixed some time before bringing them in contact with the organic compound little or no oxidation would result since iodate will not attack these compounds under the conditions used.

(5) $3NaOI \longrightarrow NaIO_3 + 2NaI$.

On acidification, all of the iodine except that used up in the oxidation of the organic compounds is converted back to free iodine.

The iodine consumed is stoichiometrically equivalent to the amount of organic compound. Since each mole of NaOI used up represents two equivalents, each mole of acetone or acetoin reacts with six equivalents and each mole of glucose with two equivalents.

This procedure is useful for checking the strengths of standards but is not so useful when applied to complex mixtures because of interference from other compounds. It may be used for determination of glucose in the presence of fructose or for the determination of acetone in the presence of certain alcohols.

Reagents: 40, 82, 89, 94, 99.

Procedure:

A five ml. aliquot of an aqueous solution of the organic compound containing less than 4 mgm. of acetone or 6 mgm. of

acetoin is mixed with 5 ml. of Normal NaOH in a 125 ml. glass-stoppered Erlenmeyer. Five ml. of 0.1 Normal iodine solution, accurately measured, is then added, the flask stoppered, shaken thoroughly and then allowed to stand at room temperature, out of contact with direct sunlight. A blank is run at the same time using 5 ml. of distilled water. After 10 min. six ml. of Normal sulfuric acid is added to each flask and the iodine titrated to the starch endpoint using Normal sodium thiosulfate of accurately known strength in the micrometer burette.

If aldoses are to be determined less alkali is used. Ten ml. of an aqueous solution containing 16 mgm. of glucose, or less, is treated with 0.5 ml. of Normal NaOH and 4 ml. of 0.1 N iodine. After acidifying with 2 ml. of Normal sulfuric acid the excess iodine is estimated by titration, as above.

Calculation:

$$\frac{(\text{Titer of blank} - \text{titer of sample}) \times \text{micrometer factor} \times \text{normality of thio}}{6} = \text{millimoles of acetone or acetoin}$$

When aldoses are being estimated divide by 2 rather than 6.

PROCEDURE 8. Oxidation by Acid Dichromate followed by Measurement of the Excess Dichromate

Principle:

Potassium dichromate has been used by many workers for the determination of organic compounds, particularly ethanol. It is used with aqueous sulfuric acid. Oxidations are more rapid in strongly acid media than in weaker ones. Compounds with end methyl groups can be oxidized to acetic acid if sufficient heat and acid are used; since acetic acid is not oxidized by this reagent it is found as an end product. Ethanol is smoothly oxidized to acetic acid in quantitative yields.

$$2K_2Cr_2O_7 + 8H_2SO_4 + 3C_2H_5OH \longrightarrow 3CH_3COOH + 2Cr_2(SO_4)_3 + 2K_2SO_4 + 11\,H_2O.$$

Compounds such as glycerol and glucose can be completely oxidized to carbon dioxide and water. The dichromate used in these stoichiometric oxidations can be determined by using a measured amount and determining the amount left at the end of the oxidation. This can be done iodometrically

$$K_2Cr_2O_7 + 6KI + 7H_2SO_4 \longrightarrow 4K_2SO_4 + Cr_2(SO_4)_3 + 7H_2O + 3I_2.$$

Potassium iodide is added in excess and the iodine released titrated with sodium thiosulfate. It is necessary to dilute the medium before adding the iodide since this titration does not give good results if the acid concentration is too high.

Reagents: 59, 62, 89, 94.

Procedure:

A 1-2 ml. aliquot of the aqueous solution containing 1-4 mgm. of ethanol or its equivalent, is pipetted accurately into a 250 ml. glass-stoppered Erlenmeyer flask. Ten ml. of the oxidizing mixture is syringed in, the flask stoppered and the contents mixed thoroughly. After standing 30 mins. at room temperature 100 ml. of distilled water is added and then about 0.5 - 1 gm. of KI crystals. After the iodide is dissolved the iodine is titrated using Normal sodium thiosulfate in the micrometer syringe. About 0.5 ml. of starch indicator is added near the endpoint and the titration continued until the deep color of the starch-iodine complex changes to the pale color of the trivalent chromium ion.

A blank is usually run with each set of determinations; although it is constant for each batch of the oxidizing mixture.

Calculations:

(Titer of blank - titer of sample) micrometer factor x Normality of thiosulfate = meq. of dichromate reduced.

One millimole	of	ethanol (46 mgm.)	reduces	4 meq.
"	"	"	acetoin (88 mgm.)	"	4 meq.
"	"	"	n-butanol (74 mgm.)	"	4.4 meq. (approximately)
"	"	"	isopropanol (60 mgm.)	"	2 meq.
"	"	"	diacetyl (86 mgm.)	"	2.8 meq. (approximately).

Acetone is not oxidized appreciably under the above conditions. The factors for n-butanol and diacetyl are empirical and should be determined by running standard solutions in parallel with the unknowns, if they are being measured.

PROCEDURE 9. Estimation of Bisulfite-Binding Capacity

References:

T.E. Friedemann, M. Cotonio and P.A. Shaffer - J. Biol. Chem. 73: 335 (1927).
I.M. Kolthoff and V.A. Stenger - Volumetric Analysis, Vol. I, page 213 (Interscience, 1942).

Principle:

Certain aldehydes, particularly acetaldehyde, can be conveniently determined in dilute aqueous solutions by reaction with bisulfite.

$$CH_3CHO + NaHSO_3 \rightleftharpoons CH_3.\overset{H}{\overset{|}{C}}.OH \quad \overset{|}{SO_3Na}$$

The equilibrium is greatly in favor of the bisulfite complex at acid reaction but is reversed by making the solution slightly alkaline. Thus if treated with excess of bisulfite most of the acetaldehyde is bound. The excess bisulfite can then be oxidized to sulfate by iodine with very little effect on the bound bisulfite. On addition of sodium bicarbonate or disodium phosphate the complex decomposes releasing bisulfite, equivalent to the acetaldehyde. This bisulfite may then be estimated by iodometric titration. Certain compounds such as lactic acid or 2,3-butanediol may be determined by this procedure since they can be quantitatively oxidized to acetaldehyde (by permanganate or periodate, respectively).

Reagents: 40, 74, 76, 94.

Procedure:

A five ml. aliquot of an aqueous solution containing 2 mgm. or less of acetaldehyde is treated with five ml. of 0.25 Molar sodium bisulfite in a 125 ml. Erlenmeyer flask. After standing for 15-20 mins. at room temperature it is cooled below 20°C, six or seven drops of the starch indicator are added and the excess bisulfite removed by 0.1 Normal iodine added from a 50 ml. burette. The solution is adjusted accurately to the endpoint by adding 0.1 Normal iodine from the micrometer burette. If the endpoint is overshot a drop of the bisulfite is added and the adjustment repeated. The reading of the micrometer is noted and then solid sodium bicarbonate (1-2 gm.) is added and the bisulfite titrated, as it is released, until an endpoint which lasts at least one minute is obtained. The reading of the micrometer is again noted and the number of micrometer units of iodine added calculated by subtraction.

Calculation:

Micrometer units of iodine equivalent to bound bisulfite x micrometer factor x normality of iodine divided by 2 = millimoles of acetaldehyde. One millimole of acetaldehyde = 44 mgm.

PROCEDURE 10. Determination of Fermentation Acids by Partition Chromatography.

Reference: A.C. Neish - Can. J. Res. B 27: 6 (1949).

Principle:

A mixture of butyric, propionic, acetic, formic, succinic and lactic acids is often formed in fermentation solutions. These can be extracted readily by ether (Procedure 2) and the mixture resolved into the individual acids by partition chromatography on a silica-water column. The amount of each individual acid is then determined by titration with standard alkali.

Succinic and lactic acids are difficult to separate and the procedure works well only if a precipitated silica prepared under carefully controlled conditions is used. (Acid-washed Celite can be used for separation of certain mixtures of these acids, however.) The acids are added to the column in aqueous solution since enough water is extracted with the ether from some fermentation solutions (e.g. yeast fermentations) to make it difficult to obtain a one-phase solution in an organic solvent immiscible with water, as was suggested in the above reference.

It is possible to do the chromatography without using the indicator stream by collecting fractions of predetermined size since the retention volumes are constant from one run to the next.

Fumaric acid, if present, interferes. It is mixed partly with the acetic and partly with the formic acid, hence one broad band of all three acids is obtained. Under these conditions the volatile acids can be distilled and determined in the distillate (see Procedures 4 and 11).

Reagents: 10, 11, 12, 20, 36, 37, 49, 53, 54, 70, 84.

Procedure:

Four gm. of the dry silicic acid is weighed into a 100 ml. beaker and at the same time an 0.6 gm. portion is weighed into a 50 ml. beaker. The larger silica sample is treated with 2.7 ml. of 0.01 Normal hydrochloric acid. This is mixed in thoroughly by rubbing the silica vigorously against the sides of the beaker with a stainless steel spatula. When this blending operation is finished the mixture is slurried in approximately 30 ml. of chloroform saturated with 0.01 Normal hydrochloric acid and poured into the chromatogram tube (see Figure 8). The silica is compressed into a column about 8 cm. long by placing a filter paper disc on top of it and then ramming it down with the glass plunger. The dry silica is then slurried with chloroform and packed down in a similar fashion (using another filter paper disc) on top of the wet silica.

Any excess solvent is poured out of the tube and an 0.5 ml. aliquot of an aqueous solution of the sample to be analysed

is pipetted directly on the top paper disc. Care is taken that it does not wet the walls of the column, except for one or two mm. above the top of the packing. This sample should contain 0.1 to 0.05 meq. of total organic acids and sufficient hydrochloric acid to make the solution 0.01 Normal. The dry salts obtained in Procedure 2 dissolved in 5 ml. of 0.21 Normal HCl per meq. of salt gives a suitable solution to put on the column. As soon as the sample has been absorbed into the packing a clean 50 ml. graduate is placed in position to catch the effluent solvent. A 1-2 ml. portion of chloroform is then added to the column and forced in by gentle air pressure, using the arrangement shown in Figure 8. The development is then started by filling the column with chloroform and applying sufficient air pressure to give a flow of 3-4 drops a second. The indicator stream is then adjusted (see Figure 8) so it is flowing at about one-tenth the rate of the solvent. When appreciable quantities of acid are coming out the indicator is yellow, otherwise it is red. The receiver is changed two or three ml. after the indicator drop changes from yellow to red. Fractions of a definite volume are collected and titrated even if the indicator does not show yellow, since measurable quantities of acid may escape detection. A record is kept of the amount of solvent and indicator in each fraction. The development procedure is usually as follows:

Fraction No.	Acid	Solvent	Ml. Solvent Plus Indicator
1	Butyric	$CHCl_3$	19
2	Propionic	$CHCl_3$	24
3	Acetic	BeB_5	42
4	Formic	BeB_{10}	30
5	Succinic	BeB_{25}	26
6	Lactic	BeB_{25}	35

When changing developers any solvent left in the column is decanted before the new solvent is poured in.

Each fraction is washed into a 250 ml. beaker using at least two volumes of carbon dioxide free water and then titrated with 0.01 Normal sodium hydroxide in the apparatus shown in Figure 9. It may be necessary to titrate with 0.01 Normal HCl if a large quantity of indicator solution is used. These fractions can be titrated, in order, while the development is being carried out; the whole determination should only require 1 to 1 1/2 hrs.

One of the chief sources of error lies in the titration. If insufficient water is used a water-in-oil emulsion will result and the titrations may be badly overshot. With the proper type of emulsion (oil-in-water) low results are usually recorded by analysts inexperienced in this type of titration since they do not allow enough time for the acids to extract from the solvent phase when near the endpoint. The titration

should be carried to a definite pink color which persists for at least one minute in the vigorously stirred solution.

A silica column can be used for three or four samples if flushed with chloroform and the section of packing at the top removed and replaced with dry silica (slurried in chloroform) before adding the next sample.

Calculation:

The titration figures have to be corrected for the blank acidity of the solvent and the base added in the indicator. Each ml. of indicator solution added is equivalent to 0.1 ml. of 0.01 Normal NaOH; hence this must be added to the amount of NaOH required in the actual titration. The small amount of acid in the solvents is determined by titration of 100 ml. of CO_2 free water in the presence and absence of 20 ml. of the solvent in question. The difference gives the blank correction to be used. This correction is negligible for chloroform but amounts to about 0.20 ml. for 20 ml. of BeB_{25}.

Corrected titer x ml. base x normality of base = meq. of acid in aliquot chromatographed

PROCEDURE 11. Determination of Volatile Acids by Partition Chromatography

Reference: See Procedure 10.

Principle:

See Procedure 10. Acid washed Celite may be used as the packing in place of silicic acid since lactic and succinic acid are not to be separated.

Reagents: 10, 11, 19, 20, 36, 37, 49, 53, 54, 84.

Procedure:

The column is packed as outlined in Procedure 10 using 3 gm. of Celite wet with 2.5 ml. of 0.01 Normal hydrochloric acid. A half-gram portion of dry Celite is slurried in chloroform and packed on top of the wet Celite packing. The salts of the volatile acids, prepared as outlined in Procedure 4, are dissolved in 5 ml. of 0.21 N HCl per meq. and an 0.5 ml. aliquot put on the column. The general technique of running the column and titrating the acids is the same as that outlined in Procedure 10. Four fractions are collected and titrated.

Fraction No.	Acid	Solvent	Ml. Solvent Plus Indicator
1	Butyric	$CHCl_3$	15
2	Propionic	BeB_5	19
3	Acetic	BeB_5	35
4	Formic	BeB_{10}	40

The determination can be simplified by dispensing with the indicator stream and collecting and titrating fractions of a predetermined volume.

Calculations:

As in Procedure 10.

PROCEDURE 12. Determination of 2,3-Butanediol and Glycerol by Partition Chromatography

Reference: A.C. Neish - Can. J. Res. B 28: 535 (1950).

Principle:

Mixtures of glycols and glycerol can be resolved by partition chromatography on wet Celite and each component then determined by measuring the acetaldehyde or formaldehyde formed on periodate oxidation by Procedures 17 and 18 respectively. It is possible to chromatograph a mixture of acetoin, 2,3-butanediol, 1,2-propanediol, ethanediol, glycerol and erythritol and recover each compound quantitatively in a separate fraction. Compounds such as the pentoses, hexoses and their corresponding alcohols remain on the column although they can be removed also if a strong enough solvent is used. The development scheme given below is a simple one designed to make the determinations of 2,3-butanediol and glycerol as specific as possible. There is no compound likely to interfere in the determination of 2,3-butanediol but dihydroxyacetone is partly recovered in the glycerol fraction. If present, it can be determined in a portion of this fraction by its reducing power for alkaline copper reagents and a correction applied to the glycerol figure. It would probably be possible to adapt this procedure to the determination of simple sugars such as diose, trioses and tetroses in the presence of the pentoses and hexoses.

Reagents: 13, 19, 28, 49.

Procedure:

A three gm. portion of the Celite is mixed thoroughly with 2.5 ml. of water, slurried in the ethyl acetate and packed into the chromatogram tube (see figure 8). A half-gram portion of dry Celite is also slurried in this solvent and packed on top of the wet Celite. The sample to be analysed should contain about 1 to 5 mgm. of glycerol or 2,3-butanediol (or both) in 0.5 ml. of water. This is pipetted directly into the packed dry Celite after decanting any supernatant solvent. Care is taken to prevent any getting on the walls of the chromatogram except for 1 - 2 mm. above the packing. The sample is washed in with two ml. of the ethyl acetate and the development started. Four fractions are collected in 50 ml. graduates.

Fraction No.	Compound in Fraction	Developing Solvent	Ml. of Solvent
1	Acetoin	Ethyl Acetate	20
2	2,3-Butanediol	Ethyl Acetate	31
3	Ethanediol, 1,2-propanediol	BeB_{75}	40
4	Glycerol	BeB_{75}	50

The ethyl acetate is decanted from the top of the column when changing to BeB_{75}. The volumes are measured after addition of the sample. During development care is taken to prevent air entering the packing. The developing solvents are forced through the column by air pressure at the rate of 3 - 4 drops per second. The elution volumes are quite reproducible but should be checked occasionally by analysing a standard solution.

The first fraction may be analysed for acetoin by shaking with an equal volume of water and then determining the acetoin in the aqueous phase by Procedure 19. When this is done the acetoin standards, in water, are shaken with an equal volume of ethyl acetate (saturated with water) in order to correct for the acetoin in the solvent phase. However, acetoin is usually determined on an aliquot of the neutral volatile fraction.

2,3-Butanediol is determined by analysis of the second fraction. This fraction is transferred to a beaker using 20 ml. of water and warmed on a steam bath with occasional stirring until the mixture becomes monophasic. The volume is then made to 20 ml. and a suitable aliquot taken for the determination of 2,3-butanediol by Procedure 18. The evaporation on the steam bath should not be longer than necessary since some loss of 2,3-butanediol may occur.

Glycerol is determined in the fourth fraction. This is mixed with an equal volume of water, in a 250 ml. beaker, and evaporated on a steam bath until 10 - 15 ml. of the aqueous phase remains. It is important that the amount of water added is at least equal in volume to the solvent since some glycerol may be lost if there is insufficient water. The residual aqueous phase is made to a volume of 25 ml. and a suitable aliquot analysed. for glycerol by Procedure 17.

PROCEDURE 13. Determination of Simple Aliphatic Alcohols by Partition Chromatography (n-Butanol, Isopropanol and Ethanol).

Reference: A.C. Neish, Can. J. Chemistry 29: 552 (1951).
See also Procedure 8.

Principle:

The simple aliphatic alcohols can be separated by chromatography on a Celite-water column using carbon tetrachloride and chloroform as developing solvents. Each alcohol can then be measured, in the presence of the developing solvent, by dichromate oxidation followed by measurement of the dichromate consumed. It is possible to resolve a mixture of isoamyl alcohol, n-butanol, isopropanol (or propanol), ethanol and methanol. Diacetyl and acetoin are found in the butanol and isopropanol fractions respectively and must be corrected for. Acetone is found in the butanol fraction but does not interfere since it is not oxidized by dichromate under the conditions used.

Reagents: 18,19,21,22,49,59,62,89,94.

Procedure:

The chromatogram tube is packed as shown in Figure 8 using 3 gm. of Celite 535 wet with 2.5 ml. of water for the bottom layer and 0.5 gm. of dry Celite for the top layer. These are slurried in washed carbon tetrachloride for transfer to the column and tamped down with filter papers using a glass plunger as described in Procedure 10. Excess carbon tetrachloride is decanted and 0.5 ml. of an aqueous solution, containing not more than 5, 10 or 4 mgm. of n-butanol, isopropanol or ethanol respectively, is pipetted onto the top of the packing, care being taken to prevent any of it from getting on the walls of the tube except for 1 - 2 mm. about the top of the filter paper. While this is being absorbed a clean, empty 50 ml. graduate is put in position to catch the eluate. The sample is washed in with two approximately one ml. portions of the carbon tetrachloride and the column developed, three fractions being collected.

Fraction No.	Developing Solvent	Ml. solvent collected	Compounds in Fraction
1	CCl_4	20	n-butanol, diacetyl
2	CCl_4-$CHCl_3$ (50%)	23	n-propanol, isopropanol, acetoin
3	$CHCl_3$	25	ethanol

The developing solvent is forced through the column by gentle air pressure at the rate of 4-5 drops per second. When changing solvents any excess of the old solvent is decanted before the new solvent is poured in.

Each fraction is transferred to a separate 250 ml. glass stoppered Erlenmeyer flask using 5 - 6 ml. of the CCl_4 to wash out the graduate. Exactly ten ml. of the acid dichromate oxidation mixture is then added to each using a syringe fitted with a stop. The flasks are stoppered and allowed to stand 30 mins. at room temperature with frequent shaking to mix the two phases. Each flask should be shaken thoroughly (for 10 seconds)

at least five times during this period. One hundred ml. of distilled water is then added to each and, after mixing, about one gm. of KI crystals. The iodine released is titrated with Normal thiosulfate using the micrometer syringe. Starch indicator is added near the end point and the flask stoppered and shaken vigorously after each addition of thiosulfate, to extract the iodine from the solvent phase. The end point is reached when the blue color of the starch-iodine complex no longer forms in the aqueous phase after 15 - 20 seconds of vigorous shaking. Blanks containing the proper amounts of the developing solvents are run in parallel and also a butanol standard.

Calculation:

Micrometer units of thiosulfate added x micrometer factor x Normality of thiosulfate = meq. of dichromate. Meq. dichromate in blank - meq. dichromate in sample = meq. dichromate reduced. One meq. dichromate reduced is equivalent to 11.5 mgm. of ethanol, 30 mgm. of isopropanol or 22 mgm. of acetoin. This follows from the fact that ethanol and acetoin are oxidized stoichiometically to acetic acid and isopropanol to acetone. Since propanol, n-butanol and diacetyl are not oxidized according to any simple equation it is necessary to run standard samples in parallel with the "unknowns" in order to obtain the proper factor. Usually one meq. of dichromate reduced is equivalent to about 16.5 mgm. of butanol. If n-propanol is present it can be determined in fraction 2 by difference, the acetoin and isopropanol contents being known from analysis by Procedures 19 and 31.

PROCEDURE 14. Colorimetric Determination of Total Carbohydrates

Reference: D.L. Morris, Science 107: 254 (1948).

Principle:

When sugars or polysaccharides are treated with anthrone in a strong solution of sulfuric acid a blue color is obtained. The intensity of the color given by starch or cellulose is approximately the same as that which would be obtained from an equal quantity of glucose. Since glucose and fructose give practically the same intensity of color a mixture of glucose, fructose, sucrose, starch and cellulose can all be analyzed and reported as glucose. This procedure is very useful for determining the carbohydrate dissimilated during a fermentation. There is no interference from acetoin, as there is when copper reduction methods are used.

The mechanism of this color reaction is not known. However furfural and hydroxymethyl furfural both give blue colors under these conditions of about the same intensity as given by equimolar amounts of pentoses and hexoses respectively. Ordinary aliphatic aldehydes give red colors. It is probable that under the action of heat and acid furfural or hydroxymethylfurfural is formed from the carbohydrates; these aldehydes then react with the anthrone to give the characteristic blue color. The method is less sensitive for pentoses than it is for hexoses. There are no

substances known which are likely to be formed in fermentation solutions which give this reaction, other than carbohydrates. Aldonic acids and sugar alcohols do not react.

Reagents: 7, 32.

Procedure:

Four ml. of a dilute aqueous solution, containing 50 - 300 γ of glucose or its equivalent, is pipetted into a 25 mm. cuvette, then 8 ml. of the anthrone reagent is syringed in. The use of a syringe is recommended since fast, reproducible mixing is obtained which ensures that the same temperature is reached in each tube. After standing 15 min. at room temperature the per cent transmittance is determined using a wave length setting of 540 mu. The standard glucose solution is kept in the refrigerator and a portion diluted twenty times each day to obtain a standard with 100 gamma of glucose per ml. This is used to set up a series of standards containing 100, 200 and 300 gamma per tube. A set of these standards, as well as a blank, is run with each batch of determinations.

Calculation:

A graph is prepared by plotting log percent transmittance against gamma of glucose per tube. This should be a straight line passing through the origin. The amount in each unknown sample is determined by reference to this graph. Single phase semi-log paper is very convenient for preparing the graph.

PROCEDURE 15. Colorimetric Determination of Reducing Sugars

References: M. Somogyi. J. Biol. Chem. 160: 61 (1945).
N. Nelson. J. Biol. Chem. 153: 375 (1944).

Principle:

When reducing sugars are heated in an alkaline solution of cupric copper, stabilized by tartrates, the copper is reduced and precipitated as cuprous oxide. The cuprous oxide formed is proportional to the amount of reducing sugar present although the relation is an empirical one. The reduced copper can be determined colorimetrically by measuring the amount of molybdenum blue formed when an arsenomolybdate reagent is added.

Reagents: 8, 32, 92.

Procedure:

Two ml. of a dilute aqueous solution containing up to 300 gamma of glucose is pipetted into a test tube graduated at 25 ml. Two ml. of the alkaline copper reagent is added, the contents of the tube mixed thoroughly and then heated in a boiling water bath for 10 mins. The tubes are cooled in a cold water bath and

then two ml. of the arsenomolybdate reagent is mixed in and the mixture diluted to exactly 25 ml. The percent transmittance is read using a wave length of 520 mu. The sensitivity may be increased by using a wave length of 660 mu. The color is quite stable. Standards are run containing 50, 150 and 300 gamma of glucose per tube with each set of determinations.

Calculation:

When log percent transmission is plotted against gamma of glucose per tube a straight line, which passes through the origin, is obtained. This graph is plotted for the standard solutions on semi-log paper, and the amount of glucose in the unknown solutions, determined by reference to it. If other reducing sugars are determined standard solutions of the sugar in question are used.

PROCEDURE 16. Volumetric Determination of Reducing Sugars

Reference: As for Procedure 15.

Principle:

This is one of the numerous methods employing alkaline copper-tartrate solutions. The reagent is a modification of that developed by Shaffer and Somogyi. Large amounts of sodium sulfate are added to prevent re-oxidation of the titrations - a troublesome point with some copper reagents. This method is empirical, each batch of reagent must be standardized against a pure glucose solution. The test solution and the reagent are mixed in equal amounts (this ratio must be strictly adhered to) and heated for a standard time. The glucose reduces the cupric copper to Cu_2O which precipitates. The amount of Cu_2O formed is linearly related to the glucose present. The Cu_2O is determined volumetrically by the reaction.

$$2\,Cu^{+} + I_2 \longrightarrow 2\,Cu^{++} + 2I^{-}$$

The iodine is formed by adding a KI solution to the reagent, which already contains KIO_3, and then acidifying. A standard excess amount of iodine is thus formed; the amount depends on the amount of KIO_3 present which is the same in each analysis.

$$KIO_3 + 5KI + 3H_2SO_4 \longrightarrow 3K_2SO_4 + 3H_2O + 3I_2$$

The iodine not used up by the Cu^{+} is then titrated by thiosulfate and the meq. of Cu reduced then determined by subtracting this value from the blank.

Reagents: 32, 64, 90, 93, 94, 98.

Procedure:

Measure exactly 5 ml. aliquots each of a solution containing up to 3 mgm. of glucose and the copper reagent into a 30 x 200 mm. test tube, mix, close mouth of tube with a bulb and heat in a boiling water bath for exactly 15 mins. Remove, cool in a cold water bath, add 2 ml. of the KI solution, mix, syringe in one ml. of 3 N H_2SO_4 and titrate the I_2 released to the starch endpoint with the 0.2 N thiosulfate in micrometer syringe. Run a blank in exactly the same manner.

Calculation:

Titer = units req'd by blank - units req'd by test sol.
Titer x micrometer factor x Norm. of thio. = meq. of Cu reduced.

The factor converting meq. of Cu reduced to mgm. of glucose is determined using a standard glucose solution.

PROCEDURE 17. Colorimetric Determination of Formaldehyde Formed on Periodate Oxidation (Glycerol)

Reference: Marguerite Lambert and A.C. Neish. Can. J. Research, B 28: 83 (1950).

Principle:

One mole of glycerol forms two moles of formaldehyde when it is oxidized by periodic acid (see reaction 4, Procedure 5). The formaldehyde may then be determined by the well known color reaction with chromotropic acid. Since iodate and periodate interfere in the color reaction they are reduced to iodide by excess arsenite in an acid medium before the color is developed. The interference caused by formation of formaldehyde from glucose can be greatly reduced by using a short oxidation time, thus making it possible to determine glycerol accurately in the presence of ten times as much glucose if a correction is applied. 2,3-Butanediol and acetoin do not interfere appreciably. Sugar alcohols and aldonic acids interfere stoichiometrically if present; in fact the method may be applied to their determination in absence of other components.

Reagents: 23, 33, 51, 72, 97.

Procedure:

An aliquot of a diluted fermentation solution containing 0.2 to 0.8 mgm. of glycerol and not exceeding 20 ml. in volume is pipetted into a glass-stoppered volumetric flask of 100 ml. capacity. Sufficient distilled water is added to make the volume 20 ml. and then one ml. of 10 Normal sulfuric acid is mixed in. Five ml. of 0.1 Molar periodic acid solution is syringed in and exactly 5 min. later 5 ml. of the Molar arsenite is added in the same way. Any number of samples up to twenty can be run in parallel.

About twenty seconds after addition of the arsenite iodine appears in the solution and then fades; 5 - 10 min. after this the contents of the flask is made to 100 ml., mixed, and exactly one ml. pipetted into a 25 x 200 mm. Pyrex test tube. Ten ml. of the chromotropic acid reagent is then added, using a syringe, and the tube heated for 30 mins. in a boiling water bath, in diffuse light. After cooling to room temperature, the solution is poured into a 25 mm. cuvette and the percent transmittance is determined using a wave length setting of 570 mu. A blank must be run with each set of determinations, and usually a standard curve as well.

The oxidations can be run on one-tenth the above scale, in test tubes, with good results, as follows. Pipette two ml. containing 20-80 gamma of glycerol into a 19 mm. diameter test tube and add 0.1 ml. of 10 N sulfuric acid. Syringe in 0.5 ml. of 0.1 M periodic acid and, five mins. later, add 0.5 ml. of Molar sodium arsenite. Mix thoroughly and, after 10 - 15 mins., make the volume to 10 ml. by syringing in 6.90 ml. of water. The color is developed on one ml. of this exactly as described in the preceding paragraph.

Calculation:

When log percent transmittance is plotted against γ of glycerol per tube the standard solutions give a straight line passing through the origin. This line has been quite reproducible from day to day during the period of almost three years that the method has been used. Unknown samples are referred to this curve (on semi log paper) and calculated as glycerol. The value obtained should be corrected for the glucose present (determined by Procedure 14, 15 or 16). The mgm. of glucose multiplied by 0.03 and subtracted from the apparent mgm. of glycerol gives an approximation of the true glycerol content. However, this correction varies with temperature and for accurate results it should be determined by running a standard glucose sample in parallel with the dilute fermentation solutions. The glycerol standard may be replaced with one prepared from recrystallized mannitol by accurate weighing. One mole of mannitol gives two moles of formaldehyde.

PROCEDURE 18. Colorimetric Determination of Acetaldehyde Formed on Periodate Oxidation (2,3-Butanediol).

Reference: P. Desnuelle and M. Naudet. Bull. Soc. Chim. France 12: 871 (1945).

Principle:

Sodium nitroprusside gives an unstable blue color with acetaldehyde in alkaline solutions. However, this test can be made quantitative if a photoelectric colorimeter (or spectrophotometer) is used since the color can be developed in the cuvette and measured by the maximum light absorption reached at a given wave

length. Periodate and iodate do not interfere markedly hence it is possible to determine acetaldehyde formed in periodate oxidation mixtures without separation of the acetaldehyde if a standard curve is run using a solution of the substance in question. This method is very useful for the determination of 2,3-butanediol in diluted fermentation solutions. One mole of 2,3-butanediol is rapidly oxidized to two moles of acetaldehyde by periodic acid (see Reaction11, Procedure 5).

Glycerol, diacetyl and glucose do not interfere if present in amounts equal to the diol. Since one mole of acetoin gives one mole of acetaldehyde a correction must be made for it.

Reagents: 16, 51, 56, 85.

Procedure:

A sample of the diluted fermentation solution containing 50 - 400γ of 2,3-butanediol is pipetted into a 19 mm. cuvette and enough water added to make the volume 5 ml. One ml. of the periodic acid solution is then mixed in and the tube allowed to stand 30 mins. at room temperature. A blank and several standard solutions are run in parallel. The color is developed in each tube, one at a time, by rapid addition of 1.5 ml. of the piperazine solution followed immediately by 0.5 ml. ofthe freshly prepared nitroprusside solution. These are best added by syringes. The galvanometer is first set at 100% transmittance using the blank, with a wave length setting of 570 mu. Each tube is then read, one at a time, by allowing the color to develop to its maximum point with the tube in the spectrophotometer. As soon as the color starts to fade the next tube is read in the same fashion. It only requires about one minute to read each tube, with practice, since the reagent can be added to the next tube in the series before the color in the tube being read reaches its maximum. The spectrophotometer should be warmed up 5 min. prior to reading the first tube; the blanks do not give a constant reading hence it is necessary to add the piperazine and nitroprusside solutions to a fresh blank each time the 100% setting is checked.

Calculations:

When log percent minimum transmittance of standards is plotted against γ of 2,3-butanediol per tube a straight line, passingthrough the origin is obtained. The amount of diol in the sample is determined by reference to this line (or semi-log paper) the standards being run in parallel with each set of samples. A correction is made for the acetoin present. One millimole acetoin (88 mgm.) gives the same color as one-half millimole of 2,3-butanediol (45 mgm.). Acetoin can be determined by Procedure 19.

PROCEDURE 19. Colorimetric Determination of Acetoin Plus Diacetyl

References: W.W. Westerfeld - J. Biol. Chem. 101: 495 (1945).
P. Eggleton, S.R. Elsden and N. Gough - Biochem. J. 37: 526 (1943).

Principle:

When dilute solutions of acetoin or diacetyl are treated with creatine and alkaline alpha-naphthol a red color forms. The color forms more rapidly with diacetyl but, since it slowly decays, it is possible to select a reaction time for which equal weights of acetoin and diacetyl give the same intensity of color and thus the sum of both of these compounds is obtained. There are no other known volatile fermentation products which give this reaction. Certain other compounds such as amino acids modify the development of the color so it is best to use a distillate like that obtained by Procedure 3.

Reagents: 1, 25, 46.

Procedure:

A sample of solution containing 2 - 15 γ of acetoin + diacetyl is pipetted into a 19 mm. cuvette and enough water added to give a volume of 5 ml. The creatine solution (1 ml.) is then mixed in and 1 ml. of the freshly prepared alkaline α-naphthol added. The tubes are shaken and allowed to stand one hour at room temperature. The percent transmittance is then determined using a wave length setting of 530 mμ. A blank and several standards are run with each set of determinations.

Calculations:

When log percent transmittance is plotted against γ of acetoin + diacetyl per tube a straight line passing through the origin is obtained. The unknown samples are referred to this graph (use semi-log paper). The amount of acetoin present is determined by subtracting the amount of diacetyl found by the dimethylglyoxime-urea reaction (Procedure 20). If the sample analysed is a distillate the acetoin in the original solution is calculated using the distillation factor; about 53.5% of the acetoin present distills. (See Procedure 3).

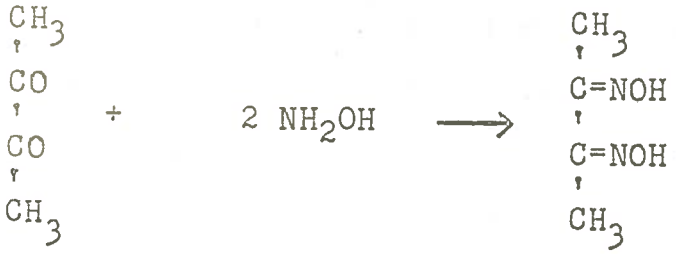
PROCEDURE 20. Colorimetric Determination of Diacetyl.

Reference: A.G.C. White, L.O. Krampitz and C.H. Werkman. Arch. Biochem. 9: 228 (1946).

Principle:

When diacetyl is treated with hydroxylamine it gives di-

methylglyoxime.



Dimethylglyoxime condenses with urea in the presence of strong acids to give a yellow compound of unknown constitution. Acetoin does not give this test, hence it is suitable for the determination of diacetyl in the presence of acetoin. It has been applied to the direct analysis of tissue filtrates (see above reference).

Reagents: 3, 26, 39, 101.

Procedure:

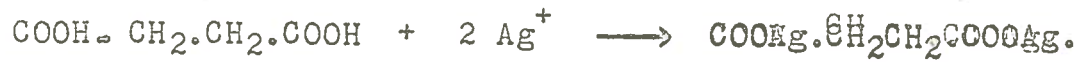
Pipette an aliquot of the volatile neutral fraction containing 15 to 60 γ of diacetyl into a 25 x 200 mm. test tube and add enough water to give a volume of 4 ml. Mix in 2 ml. of the hydroxylamine solution, 2 ml. of the urea solution and finally 4 ml. of the H_3PO_4-H_2SO_4 mixture. The tube is heated 35 min. in a boiling water bath, cooled and the percent transmittance measured in a 25 mm. cuvette using a wave length setting of 475 mu. Standard diacetyl solutions are run in parallel with the solutions being analysed.

PROCEDURE 21. Determination of Succinic Acid as the Silver Salt.

Reference: D.M. Moyle - Biochem. J. 18: 351 (1924).

Principle:

Succinic acid is stable in boiling acid permanganate solutions while the other non-volatile acids produced by fermentation are not. The solution is treated with hot acid permanganate, the succinic acid separated from the inorganic acids by ether extraction, and precipitated as the silver salt.



The silver succinate precipitates at the bromocresol purple end point. It is filtered off, washed free of soluble silver salts with 50% ethanol and dissolved in dilute nitric acid. The silver is then determined by Volhard's method. This depends on the fact AgCNS is insoluble in dilute nitric acid. The solution is titrated with a standard NH_4CNS solution

Some ferric alum is added as an indicator; when the point of equivalence is reached the reddish color of $Fe(CNS)_3$ appears. The first persistent salmon pink color is taken as the end point.

Reagents: 5, 6, 14, 27, 30, 47, 65, 71, 97.

Procedure:

The method is not sensitive and is best applied to acid fractions obtained, by large scale ether extractions as described in Procedure 2. An aliquot containing up to 75 mgm. of succinic acid in 25 ml. of water is mixed with 3 ml. of 10 Normal sulfuric acid in a 150 ml. beaker. Three glass beads are added and the solution heated to boiling and titrated with 0.1 N permanganate solution until a brown precipitate remains after the solution has been evaporated to 15 - 20 ml. Filter, cool and extract with ether in a large extractor as outlined in Procedure 2, for 24 hours. (Add 20 ml. of water to the extraction flask). Evaporate the ether on a steam bath, add 5 ml. of 10% silver nitrate, 3 drops of the bromocresol purple and titrate with 0.5 N ammonium hydroxide until the indicator assumed a muddy bluish shade; do not let it get purple since silver succinate dissolves in excess alkali. Filter through a Gooch crucible, with an asbestos mat, wash with 10 ml. of 50% ethanol, dissolve the precipitate with two 10 ml. portions of the hot dilute nitric acid (adding the solution back to the extraction flask), transfer the asbestos to the flask with water, add 2 ml. of the ferric alum indicator and titrate with 0.2 N thiocyanate solution in the micrometer syringe to the first permanent pink endpoint.

Calculation:

One mol. of succinic acid precipitates 2 equivalents of silver.

$$\frac{\text{micrometer units} \times \text{micrometer factor} \times \text{normality of thiocyanate}}{2} = \text{millimoles of succinic acid.}$$

PROCEDURE 22. Volumetric Determination of Lactic Acid

Reference: T.E. Friedemann and J.B. Graeser - J. Biol. Chem. 100: 291 (1933).

Principle:

The lactic acid is oxidized to acetaldehyde with permanganate.

$$CH_3.CHOH.COOH + O \text{ (from } KMnO_4) \longrightarrow CH_3.CHO + H_2O + CO_2$$

This is done in the apparatus shown in Figure 12. Dilute $KMnO_4$ is allowed to drop slowly into the boiling solution and the acetaldehyde distilled out as it is formed. The reaction is quantitative. The acetaldehyde is absorbed by a $NaHSO_3$ solution and determined as explained in Procedure 9. This

method is accurate but can only be used on the organic acid fraction obtained in Procedure 2 since 2,3-butanediol and glucose interfere.

Reagents: 40, 44, 66, 74, 76, 94.

Procedure:

Place 10 ml. of the H_3PO_4-$MnSO_4$ reagent in a 250 ml. long-necked flask, pipette in aliquot of the acid fraction to be analysed (1 - 10 ml.) and add enough water to make the volume to approximately 100 ml. Attach to the distillation apparatus (Figure 12), put 5 ml. of the $NaHSO_3$ soln. and 5 ml. of water in the 250 ml. Erlenmeyer used as the receiver and heat the contents of the long-necked flask to boiling. Allow 0.02 N $KMnO_4$ to drop into the boiling solution drop by drop until an excess is present. This oxidation should require 10 to 15 mins., during which about 20 -30 ml. distils over. After it is complete allow 5 mins. more then lower the flask for the last 5 ml. of distillate. Cool the receiver below 25°C., add starch indicator and titrate to determine the acetaldehyde as described in Procedure 9.

Calculation:

One millimole of acetaldehyde = 1 millimole of lactic acid.

PROCEDURE 23. Colorimetric Determination of Lactic Acid

References: S.B. Barker and W.H. Summerson, J. Biol. Chem. 138: 535 (1941).
R.L. Markus - Arch. Biochem. 29: 159 (1950).

Principles:

When a very dilute solution of lactic acid is heated in presence of a high concentration of sulfuric acid it is converted to acetaldehyde. The acetaldehyde may then be determined by the sensitive color test employing p-hydroxydiphenyl. Precautions must be taken to avoid contaminating the very dilute solutions with lactic acid from perspiration or saliva; large errors are often introduced in this way by inexperienced analysts. In the modification of this procedure given below sufficient heat is generated by rapid mixing of the sulfuric acid and water.

Reagents: 24, 38, 41, 95.

Procedure:

One ml. of the diluted fermentation solution, containing 1 - 10γ of lactic acid is pipetted into a 19 mm. cuvette and one drop (.05 ml.) of the copper sulfate solution added. Six ml. of concentrated sulfuric acid is syringed in, the tubes allowed to stand 5 mins in air and then cooled below 20°C. in cold water.

One drop (0.05 ml.) of the p-hydroxydiphenyl solution is added without touching wall of tube, mixed in thoroughly, and the tube allowed to stand 6 - 8 hrs. at room temperature (or overnight). The percent transmittance is measured using a wave length setting of 570 mu. A blank and a standard curve is run with each set of determinations. The standard curve is prepared by accurate dilution of the standard solution of lithium lactate.

Calculation:

When γ of lactic acid per tube is plotted against log percent transmittance a straight line passing through the origin is obtained. Unknown samples are referred to the graph (use semi-log paper) obtained with standards run in parallel. Glucose interferes slightly in this method. One mgm. glucose gives about the same colorimeter reading as 0.02 mgm. of lactic acid. The weight of residual carbohydrate, determined as glucose, is multiplied by 0.02 and subtracted from the weight of apparent lactic acid in the same sample to get the true amount of lactic acid.

PROCEDURE 24. Colorimetric Determination of Organic Matter.

Reference: M.J. Johnson. J. Biol. Chem. 181: 707 (1949).

Principle:

Most organic compounds if heated with dichromate in the presence of high concentration of sulfuric acid are oxidized to some extent. As pointed out on page 24 acetic acid is an exception. The reduced dichromate may be measured directly because of the green color of the trivalent chromium ion. The Beer-Lambert law is obeyed, with little interference from the dichromate color, if a wave length setting of 650 mu. is used. A solution of dichromate of known normality is used and two controls are run. One of these controls containing water and oxidizing mixture is a blank used to set the spectrophotometer at 100% transmittance. The other blank is treated with sufficient bisulfite to reduce all the dichromate. It serves as a standard, giving the percent transmittance for a known concentration of trivalent chromium.

Reagents: 76, 78.

Procedure:

Two ml. of solution containing about 0.5 - 2.5 mgm. of organic matter is pipetted into a 25 by 200 mm. test tube and 5 ml. of the sodium dichromate oxidizing mixture added, using a syringe. A control is run on the distilled water and the sodium bisulfite solution. The tubes are heated 20 mins. in a boiling water bath, cooled to room temperature and the contents

transferred to 19 mm. cuvettes. The percent transmittance is determined at a wave length setting of 650 mu, using the water control to set the galvanometer at 100 percent transmittance. The bisulfite control gives the percent transmittance corresponding to complete reduction of the dichromate.

Calculation:

The percent transmittance of the bisulfite control is usually about 20% and corresponds to about 0.5 meq. of dichromate reduced. A graph of percent transmittance versus meq. of dichromate reduced is made on single phase semi-log paper by joining this point to 100 percent transmittance at zero dichromate reduced. The meq. of dichromate reduced by the unknown samples is determined by reference to this graph. This method is useful for detecting unknown compounds in fermentation solutions. The figure obtained is corrected for the quantities of known compounds present and the difference is a measure of the unknown compounds. The following table gives experimentally determined oxidation factors for some substances likely to be found in fermentation solutions. The figures shown in brackets are the theoretical values for complete oxidation to carbon dioxide and water. Sugars and sugar alcohols are completely oxidized.

Compounds	Meq. dichromate reduced per millimole	Mgm. of compound per meq. dichromate reduced
Glycerol	13.9 (14)	6.46
Glucose	24.7 (24)	7.26
Acetoin	12.6	6.98
2,3-Butanediol	11.7	7.68
Lactic Acid	4.7	19.1
Mannitol	- (26)	7.29
Succinic Acid	4.48	26.4
Citric Acid	20.0	9.65
Formic Acid	- (2)	23.0
Butyric Acid	15.2	4.86
Malic Acid	13.5	9.94
Acetic Acid	0.088	695.
Ethanol	4.53	10.15
Acetone	4.20	13.8
Yeast Extract	-	10.3
Casein	-	5.8

PROCEDURE 25. Determination of Total Carbon in Fermentation Solutions.

References: D.D. Van Slyke and J. Folch. J. Biol. Chem. 136: 509 (1940).
M. Calvin, C. Heidelberger, J.C. Reid, B.M. Tolbert and P.F. Yankwich. Isotopic Carbon, Wiley, 1949. Chapter 6.

Principle:

Organic compounds are oxidized quantitatively to carbon dioxide if heated with the Van Slyke-Folch combustion fluid. The carbon dioxide might be determined by any one of several gravimetric, volumetric, or gasometric techniques. In the present method it is determined gravimetrically by precipitation as barium carbonate. Although a more sensitive or rapid technique might be used, this one was chosen because it permits the use of simple apparatus and also because the carbon is isolated in a form suitable for radioactivity measurements.

The method gives reliable results even with volatile compounds such as ethanol or acetone.

Reagents: 4, 9, 61, 83, 102, 104.

Procedure:

The apparatus used is illustrated by Figure 10. The sample is heated with the combustion fluid to a definite temperature and the carbon dioxide swept out with purified air through a bubbler containing acid dichromate (reagent 57) into an absorption tube containing 40 ml. of 0.25 Normal NaOH. The bubbler gives a check on the rate of flow of the air stream; acid dichromate is used to retain volatile oxides of nitrogen and sulphur. The carbon dioxide absorption tube shown in figure 10 can be replaced by the bead tower shown in Figure 11.

To perform an analysis first charge the absorption tube with 40 ml. of 0.25 Normal NaOH and connect it to the apparatus. Add 0.6 gm. of powdered KIO_3 to the combustion flask using a Van Slyke scoop and then pipette in 0.5 to 2.0 ml. of the solution to be analysed (which should contain 2 to 20 mgm. of organic carbon), and connect the flask to the absorption train. Syrupy phosphoric acid is used to lubricate the glass joints connected to the combustion flask and the thermometer joint. Ten ml. of the combustion fluid is then injected through the air inlet tube of the combustion flask by a syringe, and the trap quickly connected. The combustion fluid is heated to 230 - 250°C. When this temperature is reached a slow stream of air (two bubbles per second) is passed through the apparatus. After 10 min. the heating is discontinued but the carbon dioxide is swept out for 5 mins. using a more rapid air stream. The absorption tube is then lowered with the air stream still passing and the gas dispersion tube rinsed with carbon dioxide free water. Five ml. of 4 Molar

[illegible] chloride is mixed with the alkali and then five ml. of [illegible] barium chloride added to precipitate the carbonate. After standing 3 to 5 mins. the barium carbonate is transferred quantitatively to a tared sintered glass crucible, washed thoroughly with hot distilled water, dried at 120°C and weighed. The weight is corrected for the appreciable amount of barium carbonate obtained in Blank runs.

Calculation:

(Wt. of $BaCO_3$ - Wt. of $BaCO_3$ given by blank x .06085 = Wt. of carbon in sample.

PROCEDURE 26. Determination of Carbonate by Titration

Reference: E.C. Conway. Microdiffusion Analysis and Volumetric Error. Crosby Lockwood and Son. 1947.

Principle:

Carbon dioxide generated in fermentations can be swept out into a bead tower and absorbed in an excess of carbonate-free NaOH.

$$2\ NaOH + CO_2 \longrightarrow Na_2CO_3 + H_2O$$

It is important to have enough excess sodium hydroxide to prevent any appreciable amount of bicarbonate from forming. If twice the amount required by the above equation is added the results should be satisfactory. An excess of $BaCl_2$ is added to precipitate the carbonate.

$$Na_2CO_3 + BaCl_2 \longrightarrow BaCO_3\downarrow + 2\ NaCl$$

The solution can then be titrated to the thymolphthalein (or phenolphthalein) endpoint since barium carbonate does not react with acids at this pH as sodium carbonate does.

Sodium hydroxide is more convenient to use than barium hydroxide since no precipitate is formed during the absorption of carbon dioxide and since stronger solutions may be used. The solutions can be washed readily from the tower before precipitation of the carbonate.

Reagents: 9, 34, 82, 100, 104.

Procedure:

The carbon dioxide formed in small scale fermentations can be determined as follows. A bead tower (Figure 11) is charged with 20 meq. of carbonate-free NaOH, accurately measured, and the

volume adjusted with carbon dioxide-free water so the liquid fills the bulb of the tower half way when the apparatus is in operation. The flask to be swept out (see page 10), which represents 0.5 ml. of fermented glucose, is connected to the inlet of the tower and the carbon dioxide swept out as described on page 11. When the sweeping is finished the alkali is washed from the tower into the flask with carbon dioxide-free water, 10 ml. of the barium chloride solution added and also 4 - 5 drops of the thymolphthalein solution. The mixture is titrated until the blue color of the indicator just disappears using 5 N HCl in the micrometer syringe. A blank is run for each new batch of reagents. Smaller amounts of carbon dioxide can be easily estimated using smaller amounts of reagents. The strength of the acid is determined by titration against the NaOH.

Calculation:

(Titer of blank - titer of sample) micrometer factor x normality of acid = meq. excess NaOH.

$$\frac{\text{meq. NaOH added - meq. excess NaOH}}{2} = \text{Millimoles of carbon dioxide}$$

PROCEDURE 27. Determination of Ethanol by Microdiffusion

References: T.H. Wennick - Ind. Eng. Chem. Anal. Ed. 14: 523 (1942).

E.J. Conway - Microdiffusion Analysis and Volumetric Error - (Crosby Lockwood and Son - 1947).

Principle:

Ethanol diffuses from the outer well of a Conway microdiffusion unit into acid dichromate in the centre well. It is oxidized to acetic acid by the dichromate and the amount present calculated from the dichromate reduced. 2,3-Butanediol and acetone do not interfere but there is a small interference from acetoin and diacetyl, if present.

Reagents: 57, 63, 91, 94.

Procedure:

Pipette exactly one ml. of the acid dichromate solution into the centre well of a Conway No. 1 microdiffusion unit. The lid is greased with vaseline and placed lightly in position. One ml. of a diluted fermentation solution,

containing 0.1 to 0.4 mgm. of ethanol, is added to the outer well and the unit sealed immediately. After standing overnight (1½ hr. or more) at room temperature the unit is opened, 1 - 2 ml. of water added to the centre well and then approximately 0.5 ml. of the 50% KI solution. The iodine released is titrated at once using 0.1 Normal sodium thiosulfate in the micrometer syringe. Two drops of starch indicator are added when near the endpoint and the titration continued until the deep color of the starch iodine complex just disappears. A blank is run with each set of determinations.

Calculation:

(Titer of blank - titer of test solution) micrometer factor x Normality of thio = meq. of dichromate reduced.

$$\frac{\text{meq. of dichromate reduced}}{4} = \text{Millimoles of ethanol}$$

meq. of dichromate reduced x 11.5 = mgm. of ethanol

PROCEDURE 28. Determination of 2,3-Butanediol by Microdiffusion

References: T.H. Winnick - J. Biol. Chem. 142: 401 (1942).

P. Desnuelle and M. Naudet - Bull. Soc. Chim. France 13: 871 (1945).

Principle:

2,3-Butanediol is oxidized to acetaldehyde by periodic acid (see equation 1, Procedure 5). If the oxidation is carried out in a Conway microdiffusion unit the acetaldehyde can be trapped in the centre well by sodium bisulfite. It may then be determined by titration of the bound bisulfite, (see Procedure 9) or by the specific color test given with sodium nitroprusside and piperazine (see Procedure 18). Since formaldehyde diffuses much more slowly the presence of an amount of glycerol equal to 2,3-butanediol does not cause much error by either method. However, if the glycerol is greatly in excess of the 2,3-butanediol (as in yeast fermentations) an appreciable error will be introduced into the volumetric measurement of the acetaldehyde. In this case the more specific colorimetric method will give reliable results, being suitable for the determination of 2,3-butanediol in the presence of 50 times as much glycerol. The only substances likely to cause errors are those giving

acetaldehyde on periodate oxidation, e.g. threonine.

The mixture is buffered during the periodate oxidation to prevent the SO_2, which back diffuses, from binding acetaldehyde in the outer well.

Reagents: 16, 40, 50, 76, 87, 88, 94 (volumetric procedure)

16, 40, 50, 56, 75, 76, 85, 88, 91, 94 (colorimetric procedure).

Procedure:

(1) Volumetric Method

Put 1 - 2 ml. sample of a fermentation solution containing 0.2 to 1.0 mgm. of 2,3-butanediol into the outer well of a Conway No. 1 microdiffusion unit (by pipetting). One ml. of sodium bisulfite is added to the centre well and one ml. of 0.38 Molar Na_3PO_4 to the outer well. The lid is greased with vaseline and the unit sealed except for a slit at the edge. The unit is tilted on a spare lid and one ml. of 0.4 Molar periodic acid is added quickly to the outer well through the slit and the unit sealed and rotated to mix the contents of the outer well. After standing 4 - 5 hrs. at room temperature, 2 - 3 drops of starch indicator are added to the centre well and the solution brought just to the blue endpoint using 0.1 Normal iodine from a micrometer syringe. The reading of the micrometer is then noted, solid Na_2HPO_4 is added to discharge the blue color and the titration with iodine continued until a stable endpoint is again reached. The volume of iodine solution used between these two endpoints is equivalent to the 2,3-butanediol present. If it is difficult to reach an endpoint it may be due to interference from formaldehyde (formed from glycerol or sugar derivatives) and the colorimetric method should be used.

(2) Colorimetric Method

The microdiffusion procedure is the same as for the volumetric procedure except only about 0.1 to 0.3 mgm. of 2,3-butanediol is added per unit. When the diffusion is finished the unit is tilted on a spare lid and the contents of the centre well transferred quantitatively to a 19 mm. cuvette, marked at 10 ml., using a bulb pipette or a small syringe to suck the liquid out of the centre well. Wash water is added from a medicine dropper, four or five washings with about 0.5 of water being required. Starch indicator (2 - 3 drops) is added to the cuvette and the endpoint adjusted to a blue color by adding 0.1 Normal iodine solution dropwise; 0.3 ml. of 6.8% $NaHCO_3$ is added and then more iodine if necessary. Sodium thiosulfate (0.1 N) is added dropwise with shaking until the solution is perfectly colorless. The contents of the tube is made to the 10 ml. mark and the

acetaldehyde determined colorimetrically as outlined in Procedure 18, with the difference that 2.5 ml. of the piperazine solution and 0.85 ml. of the sodium nitroprusside solution is used. A calibration curve is prepared from a standard 2,3-butanediol solution and run in parallel with the solutions being analysed. A straight line passing through the origin is obtained when log percent transmission is plotted against the γ of diol per tube (or unit).

PROCEDURE 29. Colorimetric Determination of Acetone

Reference: F.D. Snell and C.T. Snell. Colorimetric Methods of Analysis - Chapter VI - D. Van Nostrand Co. (1937).

Principle:

Acetone reacts with salicylaldehyde in alkaline solution to form a deeply colored orange product. The reaction is fairly specific for acetone although butanone also gives a color. Acetoin and diacetyl, in concentration equal to the acetone, do not interfere.

Reagents: 2, 69, 79.

Procedure:

A sample of the neutral volatile fraction containing 10 - 40 γ of acetone is pipetted into a 25 x 200 mm. test tube and sufficient water added to make the volume 5 ml. Four ml. of 10 Normal NaOH is mixed in and then one ml. of the salicylaldehyde solution. The tube is heated at 45 - 50°C for 20 mins., and then allowed to stand 30 mins. at room temperature. The percent transmittance is then measured in a 19 mm. cuvette using a wave length setting of 530 mu. Standard solutions are run in parallel with the samples being analysed.

Calculation:

A straight line passing through the origin is obtained when log percent transmittance is plotted against γ of acetone per tube. The acetone in the samples being analysed is determined by reference to this curve (use semi-log paper).

There is a slight deepening of the color if acetoin or diacetyl are present in ten times the acetone concentration. If it is desired to determine acetone under these conditions the proper amount of diacetyl or acetoin should be added to the standards.

PROCEDURE 30. Determination of Acetone by Microdiffusion

Principle:

The salicylaldehyde reaction (see Procedure 29) for the colorimetric determination of acetone can be combined with microduffusion, thus making it applicable to the direct analysis of fermented solutions, without any preliminary distillation.

Reagents: 2, 68.

Procedure:

Two ml. of the alkaline salicylaldehyde is pipetted into the inner well of a Conway No. 1 microdiffusion unit. The lid is greased with vaseline and placed lightly in position. One or two ml. of a suitable diluted fermentation solution, containing 10 to 40 γ of acetone, is added to the outer well and the unit sealed immediately. It is allowed to stand overnight at room temperature and the colored contents of the inner well transferred to a 19 mm. cuvette using a bulb pipette. Distilled water added by a medicine dropper is used to effect quantitative transfer. The contents of the cuvette is then made to the 10 ml. mark and the percent transmittance determined using a wave length setting of 530 mu. Standard acetone solutions are run in parallel with the samples being analysed.

Calculation:

When log percent transmittance is plotted against γ of acetone per unit a straight line passing through the origin is obtained. The acetone in the "unknowns" is determined by reference to this graph (use semi-log paper).

PROCEDURE 31. Determination of Acetone plus Isopropanol by Microdiffusion.

Principle:

Isopropanol can be quantitatively oxidized to acetone by acid dichromate. The acetone can then be determined after microduffision from the oxidation mixture. Any acetone originally present in the fermentation solution will be recovered quantitatively hence the sum of the two is determined. The excess dichromate is reduced by ferrous sulfate before micro-diffusion to prevent the ethanol in the salicylaldehyde reagent from being oxidized to acetaldehyde in outer well since, on diffusion back to the centre well, acetaldehyde interferes.

Reagent: 2, 31, 68, 77, 96.

Procedure:

One or two ml. of solution containing 1 to 4 mgm. of acetone and isopropanol is pipetted into a 100 ml. volumetric

flask (glass-stoppered) one ml. of 2 Molar sodium dichromate added and then 10 ml. of the 19 Normal sulfuric acid. After standing 30 mins. at room temperature the mixture is treated with 20 ml. of 18% ferrous sulfate, and made to volume with distilled water and mixed thoroughly. One or two ml. is then used for the determination of acetone by microdiffusion as described in Procedure 30. The standard curve is obtained using solutions of acetone run through both the oxidation and microdiffusion procedures.

Calculations:

One millimole of isopropanol (60 mgm.) gives one millimole (58 mgm.) of acetone.

Hence, $\left(\begin{array}{l}\text{mgm. acetone after dichromate oxidation} \\ \text{- mgm. acetone originally present}\end{array}\right) \times \frac{60}{58}$ = mgm. of isopropanol.

PROCEDURE 32. Analysis of Fermentation Gases

Principle:

The following procedure is a rapid method using a very simple apparatus. It is based on the well known Hempel method for gas analysis. In the method described below use is made of syringes for the transfer of gas samples and stopcocks are avoided by the use of serum bottle caps. The readings are significant to about 0.05 ml. Carbon dioxide and oxygen may be determined in one sample and carbon dioxide and hydrogen in another.

(a) Determination of carbon dioxide and oxygen

Reagents: 60, 67, 103.

Procedure:

The gas burette shown in Figure 13A is mounted with the serum bottle cap on top and filled with acidulated water. All gas is removed through the cap using a syringe fitted with a 24 gauge steel needle. A 30 ml. syringe fitted with a steel stopcock and needle (see Figure 13 B) is filled with the acidulated water, the water ejected and then it is filled with gas (22 - 25 ml.) from the fermentation flask (see page 10). This is easily done by inserting the needle through the serum bottle cap on the flask, opening the cock, pulling back the plunger, closing the cock and then withdrawing the needle. The gas sample is then injected into the burette through the cap. Its volume (R_1) is read after about 30 seconds drainage. The tube to the levelling bulb is then clamped off near the burette and 1.5 ml. of 30% KOH injected into the burette. After rotating the burette seven or eight

[illegible] over end), during a period of one minute it is returned to the upright position, the clamp removed and the volume (R_2) read after the walls have drained. The tube is again clamped, 4 ml. of the K pyrogallate is injected and the burette rotated for about 3 mins. It is then returned to the upright position, the clamp removed and the volume (R_3) read after about 30 secs. Since the solution is usually too dark to permit reading the bottom of the meniscus it is necessary to read the point at which the liquid meets the tube and then to add 0.20 ml. The burette is inverted and the cap removed allowing all the acidulated water to run out into a beaker. After washing all traces of alkali out the burette is filled with fresh acidulated water and is ready for the next analysis.

All readings are made with liquid in the levelling bulb at same height as that in the burette.

Calculation:

The results are expressed as % by volume. The burette used has 0.80 ml. of gas above the graduated part of the tube, therefore R_1 + 0.80 ml. = Volume of gas sample = V

$R_1 - R_2$ = ml. of CO_2 in gas sample.

$R_2 - R_3$ = ml. of O_2 in gas sample.

Therefore % $CO_2 = \frac{(R_1 - R_2)\ 100}{V}$ % $O_2 = \frac{(R_2 - R_3)\ 100}{V}$

(b) Determination of carbon dioxide and hydrogen

Reagents: 48, 60, 103.

Apparatus: (1) Gas burette, as above.

(2) Pd asbestos tube joined to Hempel reservoir pipette at one end and closed by a serum bottle cap at the other (see Figure 14). The Pd asbestos is heated and cooled several times in a test tube before being packed into the 2 mm. bore U tube. The tube is packed with a small plug of "Gooch asbestos" then about 2 - 3 cm. of Pd. asbestos and finally another plug of "Gooch asbestos". It is heated in boiling water during use.

Procedure:

The gas sample is put in the burette and the carbon dioxide determined as outlined above. Then 2 ml. of oxygen is injected (if necessary) and a reading taken. The gas is then removed quantitatively from the burette using the 30 ml. syringe fitted with a No. 24 needle and a stopcock; about 1 ml. of the liquid is withdrawn also. The gas is then pumped slowly back and forth twice through the Pd asbestos tube, care being

taken to displace the gas from the needle by the liquid. The gas is finally withdrawn until the liquid in the reservoir pipette is at the same mark as at the beginning, the stopcock closed and the needle withdrawn. The gas is then injected quantitatively back into the burette and it's volume again read, to the nearest 0.05 ml.

Calculations:

The % CO_2 is calculated as above. The hydrogen is combusted to water over the Pd. catalyst: $2H_2 + O_2 \xrightarrow[100°C]{6\ Pd} 2H_2O$ hence if the decrease in volume caused by passing the gas through the Pd tube is v then $\frac{200\ v}{3\ V}$ = % H_2 in gas sample (by volume); where V is volume of gas sample taken as above.

PROCEDURE 33. A Method of Mounting Solids for Determination of Carbon-14.

Reference: Calvin, M., Heidelberger, C., Reid, J.C., Tolbert, B.M. and Yankwich, P.F. Isotopic Carbon, Chapter 7 (John Wiley and Sons, 1949).

Principle:

Carbon-14 has been measured most frequently as $BaCO_3$ by the majority of workers. The beta particles emitted by this isotope are extremely weak, being practically all absorbed by material with a thickness corresponding to about 18 mgm. per square cm. Because of this it is often experimentally feasible to measure the activity of "infinitely thick" samples. The specific activities (conveniently reported as microcuries per gm. of carbon) are directly proportional to the counts/min. found for an infinitely thick sample; the factor of proportionality can be evaluated for any particular apparatus by counting a standard sample.

The method of mounting samples described below is suitable for use when enough material is available to make an infinitely thick mount, or one thick enough so the correction to infinite thickness (determined from a graph of weight vs. counts/min.) is not too large. The directions are given for $BaCO_3$ but certain organic compounds can be mounted quite successfully provided they can be crystallized in such a way as to obtain coherent precipitates. The mounting of solid samples by filtration has been described by a number of workers. The present method differs in using porous stainless steel as the filtering medium and in mounting the samples on paper discs rigid enough to be handled without special supports. Self absorption curves show the apparent infinite thickness to correspond to about 12 mgm./cm.^{2} for material mounted in this fashion. The curves are reproducible but are not logarithmic absorption curves; the activity increases in an almost linear fashion as more material is added and then levels off to the

"infinite thickness" value rather suddenly. Presumably this behaviour is due to the manner in which paper adsorbs $BaCO_3$.

Reagents: 4, 9.

Apparatus:

The samples are filtered on tared discs of Whatman No. 3 paper using an apparatus which ensures a constant area of deposit and leaves an annulus of bare paper around the edge. If dried under suction the paper remains flat and can be handled by forceps as if it were a dish. The sample can be labelled by writing on the annulus with lead pencil; this is usually done before filtration.

The filtration apparatus is shown in Figure 15. The filter paper disc, X, is supported on the porous stainless steel plate, Y. A stainless steel sleeve, W, is held tightly against the paper by the screw cap, U. The hole in this cap has the same inside diameter as the sleeve, about 1 - 1/8 inches, in our apparatus. Since the filter paper discs are 1 - 3/8 inches in diameter the mounts have a deposit, Z, with diameter 1 - 1/8 inches surrounded by an annulus of bare paper 1/8 inch wide. The cap U and funnel V are turned from brass. The porous stainless steel plate was obtained from a beaker filter made by the Micro Metallic Corporation, Brooklyn, N.Y. The apparatus is used as a suction filter in conjunction with a conventional filter pump.

Procedure:

The sample to be measured may be a carbonate solution obtained from fermentation (see page 11) or from combustion of organic matter (Procedure 25). This solution should contain 0.1 - 0.2 Normal NaOH in excess, otherwise the precipitate formed may be too granular to form a cohesive mount. The precipitation is carried out in a glass stoppered flask by addition of 3 ml. of 4 Molar ammonium chloride followed by 3 ml. of Molar $BaCl_2$ to 50 ml. of the sample containing about 0.2 - 0.5 mM of sodium carbonate. After standing 10 - 15 mins. the precipitate is filtered off using the apparatus described above, washed four times with water, then with alcohol and finally with ether. After drying 5 mins. under suction the cap and sleeve are removed, the porous plate is raised by inserting a glass rod up the funnel stem, the paper disc removed by forceps, and then dried 5 mins. more under a heat lamp. A short brass cylinder with an inside diameter about 1/8 inch greater than the diameter of the deposit is used to hold down the edges of the paper while under the heat lamp. The air-dry paper discs are tared before assembling the filter and again after drying the mounts. The activity of the dried and weighed sample is then measured. This method of mounting gives deposits of constant area and leaves an annulus of bare paper around the edge which is convenient for marking and handling the samples. The weights are significant within about 2 mgm; the activities of different mounts made from the same sample do not usually differ by more than four percent.

PART V. SOLUTIONS AND REAGENTS

1. Acetoin Standard

Crystalline polymerized acetoin can be obtained from N.V. Nederlandsche Gist-en Spiritusfabriek, Delft, Holland. Approximately one gm. is dissolved in 200 ml. of water and an aliquot standardized by Procedure 5 or 7. Store in refrigerator.

2. Acetone Standard

Dissolve one ml. of reagent grade acetone in 200 ml. of water. An aliquot is standardized by Procedure 7. Store in a glass stoppered flask in a refrigerator.

3. Acid Mixture for Diacetyl Determination

Pour 100 ml. of concentrated sulfuric acid into 300 ml. of syrupy (85%) phosphoric acid. Store in a glass stoppered bottle.

4. Ammonium Chloride (4 Molar)

Dissolve 107 gm. of ammonium chloride in water and dilute to a volume of 500 ml.

5. Ammonium Hydroxide (0.5 Normal)

Mix 9 ml. of concentrated ammonium hydroxide with water and adjust the volume to 250 ml.

6. Ammonium Thiocyanate (0.2 Normal)

Dilute "Acculute" standard for one liter of 0.100 Normal to exactly 500 ml. in a volumetric flask.

7. Anthrone Reagent

Anthrone is now available commercialy. Dissolve 2 gm. of anthrone crystals in one liter of 95% sulfuric acid (prepared by cautions addition of one liter of concentrated sulfuric acid to 50 ml. of water).

8. Arsenomolybdate Reagent

Dissolve 25 gm. of ammonium molybdate in 450 ml. of distilled water and then stir in 21 ml. of concentrated sulfuric

acid. Add 3 gm. of $Na_2HAsO_4 \cdot 7H_2O$ dissolved in 25 ml. of water and incubate the mixture at 37°C for two days. This reagent is fairly stable if filtered and stored in a brown glass bottle.

9. Barium Chloride (Molar)

Dissolve 104 gm. of barium chloride (or 122 gm. of $BaCl_2.2H_2O$) in water and dilute to 500 ml. If it is to be used for Procedure 26 neutralize to the thymolphthalein end-point with normal NaOH before dilution.

10. BeB_5 for Chromatography of Acids

Mix 20 ml. of n-butanol (reagent grade) with 380 ml. of benzene. Shake the mixture with 80 ml. of 0.01 Normal HCl in a separatory funnel for 2 min. Separate the top layer and filter it through a dry filter paper.

11. BeB_{10} for Chromatography of Acids

Mix 40 ml. of n-butanol with 360 ml. of benzene and saturate with 0.01 Normal HCl as described for reagent 10.

12. BeB_{25} for Chromatography of Acids

Mix 100 ml. of n-butanol with 300 ml. of benzene and saturate with 0.01 Normal HCl as described for reagent 10.

13. BeB_{75} for Chromatography of Glycerol

Mix 100 ml. of benzene with 300 ml. of n-butanol and shake the mixture with 100 ml. of distilled water in a separatory funnel. Separate the upper layer and filter it through a dry paper.

14. Bromocresol Purple (0.02%)

Grind 50 mgm. of bromocresol purple powder with 18.5 ml. of 0.01 Normal NaOH, in a mortar, and dilute to 250 ml.

15. 2,3-Butanediol (10%)

The diol used should be free of acids and acetoin. Prepare just before use by diluting 2,3-butanediol with nine volumes of water.

16. 2,3-Butanediol Standard

Syringe one ml. or redistilled 2,3-butanediol into 200 ml. of water. Standardize by Procedure 5 and store in a glass-stoppered flask in the refrigerator.

17. Butanol Standard

Pipette one ml. of reagent grade n-butanol into a tared, glass-stoppered volumetric flask of 100 ml. capacity. Re-weigh the stoppered flask to determine the weight of butanol added, to the nearest milligram. Adjust the volume to 100 ml. with water, mix thoroughly and store in the refrigerator.

18. Carbon Tetrachloride for Chromatography of Alcohols

Reagent grade carbon tetrachloride (400 ml.) is washed twice with 400 ml. of distilled water in a separatory funnel and then filtered through a dry paper.

19. Celite (Acid-washed)

Slurry Celite 535 with contentrated HCl and allow to stand at room temperature overnight. Wash twice with water by decantation and then on a Buchner funnel until free of acid. Wash with alcohol and then ether, dry first in air and then at 150°C overnight. This Celite is suitable for the separation of acids, alcohols and polyols by partition chromatography; recoveries of acids are poor if the Celite is not treated as above.

20. Chloroform for Chromatography of Acids

Shake 400 ml. of reagent grade chloroform with 80 ml. of 0.01 Normal HCl in a separatory funnel for 2 mins. Separate the lower layer and filter through a dry filter paper. Prepare fresh each day it is used.

21. Chloroform for Chromatography of Alcohols

The ethanol usually added as a preservative is removed by washing reagent grade chloroform six times in a separatory funnel, using an equal volume of water for each washing. The purified chloroform is filtered through a dry paper. Make up fresh each day it is used.

22. Chloroform - Carbon Tetrachloride (50-50)

Mix equal volumes of washed chloroform (reagent 21) and

washed carbon tetrachloride (reagent 18) and filter the mixture through a dry filter paper. Make up fresh each day it is used.

23. Chromotropic Acid Reagent

Dissolve one gm. of Eastman's 1,8-dihydroxynapthalene-3,6-disulfonic acid (chromotropic acid) in 100 ml. of distilled water and filter. Add 300 ml. of concentrated sulfuric acid to 150 ml. of water, cool, and add to the sulfonic acid solution to make 500 ml. Store in a brown bottle; prepare fresh every 2 - 3 weeks.

24. Copper Sulfate (4%)

Dissolve 4 gm. of $CuSO_4.5H_2O$ in water and adjust the volume to 100 ml.

25. Creatine Solution (0.5%)

Dissolve one gm. of creatine in water and adjust the volume to 200 ml.

26. Diacetyl Standard

Dissolve one ml. of commercial diacetyl in 100 ml. of water. Distil the solution and dilute the first 25 ml. of distillate to 200 ml. Determine the strength of this solution by Procedure 5: store it in a glass stoppered flask in the refrigerator.

27. Ethanol (50%)

Mix equal volumes of ethanol and water.

28. Ethyl Acetate for Chromatography

Shake reagent grade ethyl acetate with one-fifth volume of water in a separatory funnel. Separate the upper layer and filter it through a dry paper. Prepare fresh each day it is used.

29. Ethyl Ether.

Any commercial brand of reagent grade.

30. Ferric Ammonium Sulfate

A saturated solution in water.

31. Ferrous Sulfate (18%)

Dissolve 36 gm. of $FeSO_4.7H_2O$ in water and adjust the volume to 200 ml. Prepare fresh each day it is used.

32. Glucose Standard

Dissolve 0.500 gm. of reagent grade anhydrous D-glucose in water and adjust the volume to exactly 250 ml. Store in the refrigerator. The strength of this solution can be checked by Procedure 7, but this is not usually done.

33. Glycerol Standard

Syringe one ml. of reagent grade glycerol into 200 ml. of water. Determine the strength of this solution by Procedure 5; store in refrigerator. This standard may be replaced with a solution of D-mannitol prepared by accurate weighing. One gm. of glycerol gives the same amount of formaldehyde, on periodate oxidation, as 1.98 gm. of mannitol.

34. Hydrochloric Acid (5 Normal)

Mix 420 ml. of concentrated HCl with water and adjust the volume to one liter.

35. Hydrochloric Acid (Normal)

An accurate solution can be readily prepared from an "Acculute" ampoule. It may also be prepared by mixing 84 ml. of concentrated HCl with sufficient water to give one liter. This solution can then be standardized against the Normal NaOH using methyl red indicator.

36. Hydrochloric Acid (0.21 Normal)

Mix 105 ml. of the Normal HCl (reagent 35) with water and adjust the volume to exactly 500 ml.

37. Hydrochloric Acid (0.01 Normal)

Pipette 10 ml. of reagent 35 into a one liter volumetric flask and dilute to the mark with water.

38. p-Hydroxydiphenyl

Dissolve 1.0 gm. of Eastman's p-hydroxydiphenyl in 100 ml. of 0.08 Normal NaOH. Store in a brown bottle in the refrigerator.

39. Hydroxylamine Hydrochloride (2.1%)

Dissolve 4.2 gm. of hydroxylamine hydrochloride in 200 ml. of water.

40. Standard Iodine (0.1 Normal)

Weigh out 12.7 gm. of resublimed iodine, using a rough balance, and transfer it to a 125 ml. glass-stoppered Erlenmeyer flask, previously tared to the nearest milligram. Stopper the flask and reweigh it on the analytical balance to determine the weight of iodine accurately. Add 40 gm. of KI crystals and 25 ml. of water; shake until all the iodine is in solution. Transfer quantitatively to a one-liter volumetric flask and make to volume. Store in a brown, glass-stoppered bottle. The normality is calculated from the weight of iodine.

12.692 gm. iodine gives a 0.1000 Normal solution.

41.

41. Lactic Acid Standard

260 mgm. of pure dry Li lactate (reagent 42) is dissolved in water and made to 250 ml. One ml. of this solution is equivalent to 1,000 γ of lactic acid. It is stored in a refrigerator and made up fresh each month. To obtain a calibration curve for Procedure 23 dilute an aliquot of this ten times and then dilute 1, 3 and 5 ml. portion of this to 100 ml. One ml. of these final dilutions contains 1, 3 and 5 γ respectively.

42. Li lactate

May be conveniently prepared following Hillig's directions (J. Assoc. Off. Agr. Chem. 25: 255 (1942)). One volume of syrupy lactic acid is mixed with two volumes water, boiled, and lithium carbonate added to the boiling solution in small portions until slightly alkaline to phenol red. The solution is then made slightly acid by addition of more lactic acid and evaporated in a steam bath until Li lactate starts to crystallize. Five volumes of ethanol are then added and the mixture allowed to crystallize in a refrigerator overnight. The crystals are filtered, using suction, washed with alcohol and recrystallized from a mixture of alcohol and water. They are dried at 100 - 105°C. This salt has no water of crystallization.

106.6 mgm. = 100 mgm. of lactic acid.

43. Magnesium Sulfate

Reagent Grade $MgSO_4.7H_2O$

44. Manganous Sulfate Reagent

Dissolve 100 gm. of $MnSO_4.4H_2O$ in 500 ml. of warm water, add 25 ml. of 85% phosphoric acid, cool and dilute to one liter.

45. Methyl Red Solution

Dissolve 250 mgm. of methyl red powder in 500 ml. of 50% ethanol.

46. α-Napthol Reagent

Dissolve one gm. of α-napthol crystals in 20 ml. of 2.5 Normal NaOH immediately before use. This solution is quite unstable and must be made up for each set of determinations. Some samples of commercial α-napthol are pure enough to use directly but others must be purified by distillation at 2 - 3 mm. pressure in a nitrogen atmosphere.

47. Nitric Acid (Dilute)

Mix one volume of concentrated nitric acid with four volumes of water.

48. Palladium Asbestos

A commercial preparation (Eimer and Amend) containing 5% palladium.

49. Paper Discs for Chromatogram Tubes

Schleicher and Schuell No. 740-E filter paper discs sold for the assay of penicillin (12.7 mm. diameter). These fit the chromatogram tube shown on figure 8.

50. Periodic Acid (0.4 Molar)

Dissolve 9.2 gm. of H_5IO_6 (G.F. Smith Chemical Co.) in water and adjust the volume to 100 ml. Store in a brown glass-stoppered bottle.

51. Periodic Acid (0.1 Molar).

Dissolve 4.6 gm. of H_5IO_6 (G.F. Smith Chemical Co.) in 200 ml. of water. Store in a brown glass-stoppered bottle.

52. Phenolphthalein Solution (0.1%)

Dissolve 200 mgm. of phenolphthalein powder in 200 ml. of 50% ethanol.

53. Phenol Red (Alkaline)

Mix 100 ml. of 0.05% phenol red (reagent 54) with one ml. of Normal NaOH and dilute to one liter with carbon dioxide free water (reagent 104).

54. Phenol Red (0.05%)

Grind 100 mgm. of phenol red powder in a mortar with 28.5 ml. of 0.01 Normal NaOH and dilute to 200 ml. with water.

55. Phosphoric Acid (Syrupy)

Reagent grade 85% phosphoric acid.

56. Piperazine Reagent

Dissolve 25 gm. of piperazine hexahydrate in 99 ml. of water and then add 1.3 ml. of 87% formic acid or its equivalent.

57. Potassium Dichromate for Microdiffusion Analysis

Dilute 25 ml. of reagent 58 to 200 ml. using 10 Normal sulfuric acid (reagent 97).

58. Potassium Dichromate (0.4 Normal)

Dissolve 9.808 gm. of dry potassium dichromate in enough 10 Normal sulfuric acid (reagent 97) to give a volume of 500 ml.

59. Potassium Dichromate Oxidizing Mixture

Dilute 100 ml. of reagent 58 to one liter using 19 Normal sulfuric acid (reagent 96).

60. Potassium Hydroxide (30%)

Dissolve 150 gm. of KOH in water and adjust the volume to 500 ml. when cool. Store in an aspirating bottle fitted with a soda-lime, the liquid outlet being plugged with a serum bottle cap.

61. Potassium Iodate

Powdered reagent grade potassium iodate.

62. Potassium Iodide

Reagent grade KI, fine crystals.

63. Potassium Iodide (50%)

Dissolve 25 gm. of KI crystals in water and adjust the volume to 50 ml.

64. Potassium Iodide (2.5%)

Dissolve 5 gm. of KI crystals and a "knife-tip" of Na_2CO_3 in water and adjust the volume to 200 ml.

65. Potassium Permanganate (0.1 Normal)

Dissolve 3.3 gm. of $KMnO_4$ in one liter of water, let stand one day and then filter through a sintered glass funnel or a layer of asbestos.

66. Potassium Permanganate (0.02 Normal)

Dilute reagent 65 five times just before use.

67. Potassium Pyrogallate

Dissolve 10 gm. of pyrogallic acid (pyrogallol) in 40 ml. of water; dissolve 240 gm. of KOH in 160 ml. of water. Cool the KOH solution to room temperature, mix with the pyrogallic acid and store immediately in an aspirating bottle under a layer of mineral oil at least 2 cm. thick. The mouth of the aspirating bottle is fitted with a soda-lime tube and the liquid outlet is plugged by a serum bottle cap.

68. Salicylaldehyde (Alkaline)

Mix one volume of reagent 69 with four volumes of 5 Normal NaOH; prepare fresh each day it is used.

69. Salicylaldehyde Solution

Dissolve one volume of salicylaldehyde in four volumes of ethanol (commercial absolute), filter if necessary. This reagent should be prepared fresh each day it is used.

70. Silicic Acid (Silica)

Mallinckrodt's Silicic Acid, "specially prepared for chromatography by the method of Ramsey and Patterson". It is essential that this brand be used.

71. Silver Nitrate (10%)

Dissolve 50 gm. in water, dilute to 500 ml. and store in a brown glass bottle.

72. Sodium Arsenite (Molar)

Dissolve 45 gm. of sodium hydroxide pellets and 100 gm. of arsenious oxide (arsenic trioxide) in water and adjust the volume to one liter.

73. Sodium Arsenite (0.11 Normal)

Add 55 ml. of reagent 72 and 20 gm. of solid sodium bicarbonate to about 800 ml. of water. Adjust the volume to one liter when the bicarbonate is dissolved.

74. Sodium Bicarbonate

Reagent grade powder.

75. Sodium Bicarbonate (6.8%)

Dissolve 34 gm. of sodium bicarbonate in water and adjust the volume to 500 ml.

76. Sodium Bisulfite (0.25 Molar)

Dissolve 2 gm. of sodium metabisulfite (or 2.6 gm. of sodium bisulfite) in 100 ml. of water. Prepare fresh each week it is used.

77. Sodium Dichromate (2 Molar)

Dissolve 119.2 gm. of $Na_2Cr_2O_7.2H_2O$ in water and adjust the volume to 200 ml. Store in a glass-stoppered bottle.

78. Sodium Dichromate Oxidizing Mixture

Dissolve 5 gm. of sodium dichromate in 20 ml. of water and dilute to one liter with concentrated sulfuric acid. The normality of each batch is determined by diluting 5 ml. with 100 ml. of water, adding KI crystals and titrating the iodine released (see Procedure 8).

79. Sodium Hydroxide (10 Normal)

Dissolve 400 gm. of NaOH pellets in water and adjust the volume to one liter. Store in a rubber stoppered bottle.

80. Sodium Hydroxide (5 Normal)

Dissolve 200 gm. of sodium hydroxide pellets in water and adjust the volume to one liter. Store in a rubber stoppered bottle.

81. Sodium Hydroxide (2.5 Normal)

Dissolve 100 gm. of sodium pellets in water and adjust the volume to one liter. Store in a rubber stoppered bottle.

82. Sodium Hydroxide (Normal)

Dilute the contents of one ampoule of "Acculute NaOH", to make 1.000 Normal, to one liter using carbon dioxide free water (reagent 104). Alternatively this reagent may be prepared from concentrated carbonate free NaOH as described by Kolthoff and Sandell, "Textbook of Quantitative Inorganic Analysis", page 524, and standardized against potassium acid phthalate.

83. Sodium Hydroxide (0.25 Normal)

Dilute 500 ml. of reagent 81 to two liters with carbon dioxide free water (reagent 104). Store in an aspirating bottle fitted with a soda-lime tube. The tip from which the liquid is dispensed should be protected from the air. Care should be taken not to introduce carbonate at this source. The small amount of carbonate present in the reagent is corrected for by blank determinations.

84. Sodium Hydroxide (0.01 Normal)

Dilute exactly 10 ml. of reagent 82 to one liter in a volumetric flask using carbon dioxide free water (reagent 104).

85. Sodium Nitroprusside (4%)

Dissolve 2 gm. of sodium nitroprusside crystals in 50 ml. of water. Prepare fresh each day it is used.

86. Sodium Periodate (0.1 Molar)

Neutralize a solution of 11.5 gm. of periodic acid in

400 ml. of water with Normal sodium hydroxide using methyl red indicator. Adjust the volume to 500 ml.

87. Sodium Monohydrogen Phosphate

Reagent grade $Na_2HPO_4.7H_2O$, powdered by grinding in a mortar.

88. Sodium Phosphate (0.33 Molar)

Dissolve 28.8 gm. of $Na_3PO_4.12H_2O$ in water and adjust the volume to 200 ml. Store in a rubber stoppered bottle.

89. Sodium Thiosulfate (Normal)

Dissolve 250 gm. of sodium thiosulfate crystals in one liter of water. Let stand 3 - 4 days at room temperature, filter, add 0.5 ml. of toluene and standardize against reagent 40 by titrating 5 ml. of the iodine using the thio in the micrometer syringe.

90. Sodium Thiosulfate (0.2 Normal)

Dilute 200 ml. of reagent 89 to one liter. Standardize against the iodine (reagent 40) from time to time.

91. Sodium Thiosulfate (0.1 Normal)

Dilute 100 ml. of reagent 89 to one liter. Standardize against the iodine (reagent 40) from time to time.

92. Somogyi Copper Reagent

Dissolve 28 gm. of Na_2HPO_4 (or 52.9 gm. of $Na_2HPO_4.7H_2O$) and 40 gm. of Rochelle salts in 700 ml. of water; add 100 ml. of Normal NaOH and then 80 ml. of a 10% solution of $CuSO_4.5H_2O$, with stirring. Finally add 180 gm. of anhydrous sodium sulfate and, when dissolved, make to one liter. Allow to stand two days, then decant and filter. Store in Pyrex glass-stoppered bottles.

93. Somogyi Volumetric Copper Reagent

Dissolve 25 meq. (0.898 gm.) of KIO_3 in one liter of reagent 92.

94. Starch Solution

Mix 2 gm. of soluble starch with sufficient water to make

a paste, dilute to 200 ml. and heat to the boiling point. Cool, add 0.2 - 0.4 ml. of toluene as a preservative. Store in a glass-stoppered bottle.

95. Sulfuric Acid

Concentrated sulfuric acid (C.P. grade) to be used for the colorimetric determination of lactic acid. Each new bottle of reagent should be tested on a lactic acid standard before use since some batches of acid are unsuitable.

96. Sulfuric Acid (19 Normal)

Pour one liter of concentrated sulfuric acid cautiously into one liter of water, with stirring.

97. Sulfuric Acid (10 Normal)

Pour 300 ml. of concentrated sulfuric acid cautiously into 600 ml. of water with stirring. Adjust the volume to one liter when cool.

98. Sulfuric Acid (3 Normal)

Pour 90 ml. of concentrated sulfuric acid into 800 ml. of water; adjust the volume to one liter when cool.

99. Sulfuric Acid (Normal)

Pour 30 ml. of concentrated sulfuric acid into 800 ml. of water and adjust the volume to one liter when cool. If desired this may be standardized against the Normal NaOH by titration to the methyl red endpoint.

100. Thymolphthalein Solution (0.1%)

Dissolve 100 mgm. of thymolphthalein powder in 100 ml. of 80% ethanol.

101. Urea (3%)

Dissolve 6 gm. of urea in 200 ml. of water.

102. Van Slyke-Folch Combustion Fluid

Place 25 gm. of chromium trioxide, 5 gm. of powdered KIO_3 and 167 ml. of 85% phosphoric acid in a one liter glass-stoppered flask. Add 333 ml. of fuming sulfuric acid (20% SO_3). Heat

the mixture with stirring to 150°C, cool with a beaker inverted over the mouth, insert stopper when cool and replace inverted beaker to keep dust off the lip.

103. Water (Acidulated)

Add 0.2 - 0.3 ml. of concentrated sulfuric acid to one liter of water and also enough methyl red to give it a definite color.

104. Water (Carbon Dioxide Free)

Aerate with carbon dioxide free air using an apparatus similar to that shown in figure 9.

105. Zinc Sulfate Solution (25%)

Dissolve 250 gm. of $ZnSO_4.7H_2O$ in one liter of water. Determine the strength of this solution, in terms of Normal NaOH by dilution of 10 ml. of 100 ml. followed by titration to the first pink color using phenolphthalein as indicator.

NOTE: Distilled water was used in the preparation of these reagents whether specified or not.

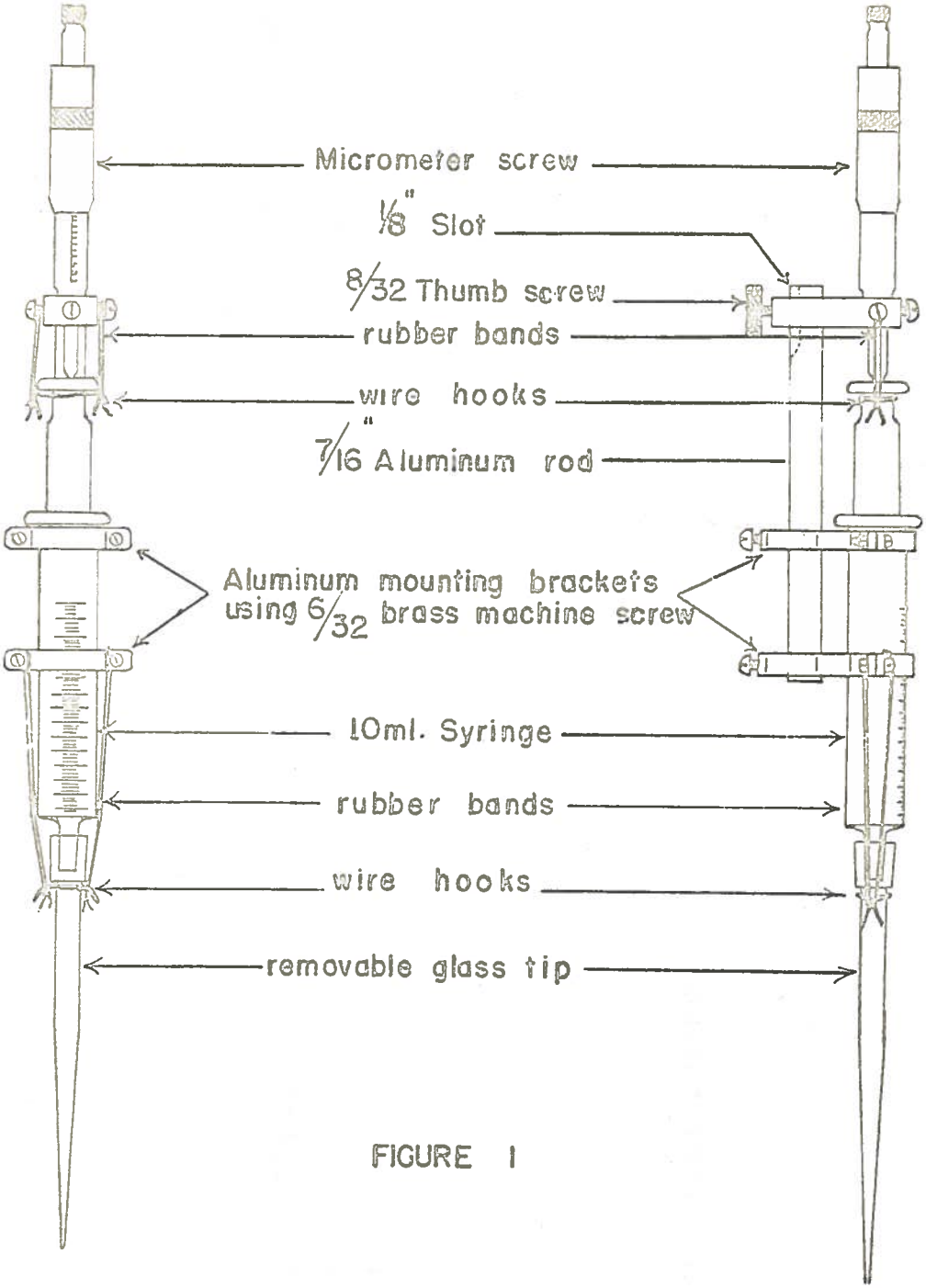

Micrometer screw
1/8" Slot
8/32 Thumb screw
rubber bands
wire hooks
7/16" Aluminum rod
Aluminum mounting brackets
using 6/32 brass machine screw
10ml. Syringe
rubber bands
wire hooks
removable glass tip

FIGURE 1

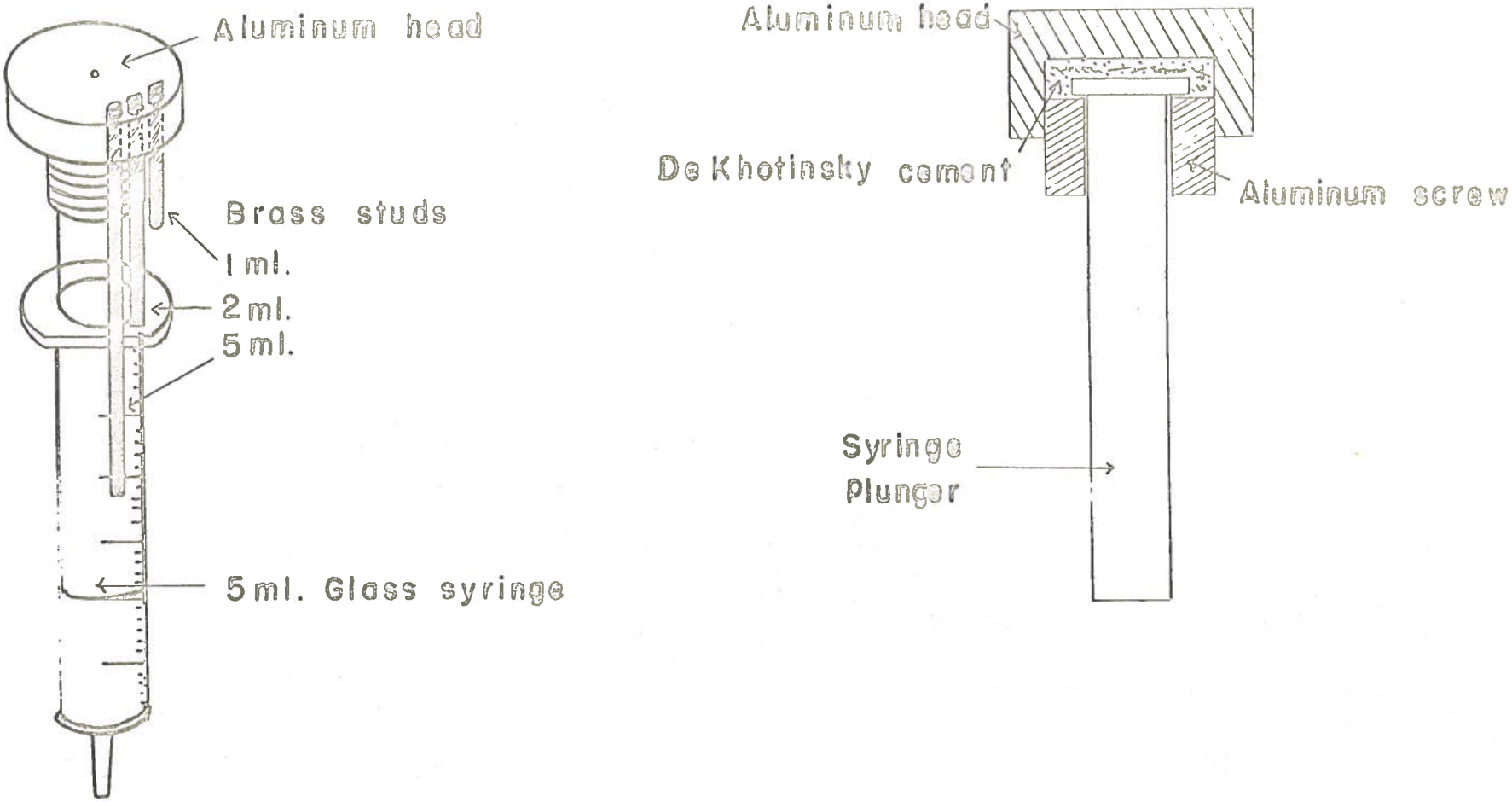

Aluminum head
Brass studs
1 ml.
2 ml.
5 ml.
5 ml. Glass syringe
Aluminum head
De Khotinsky cement
Aluminum screw
Syringe
Plunger

FIGURE 2

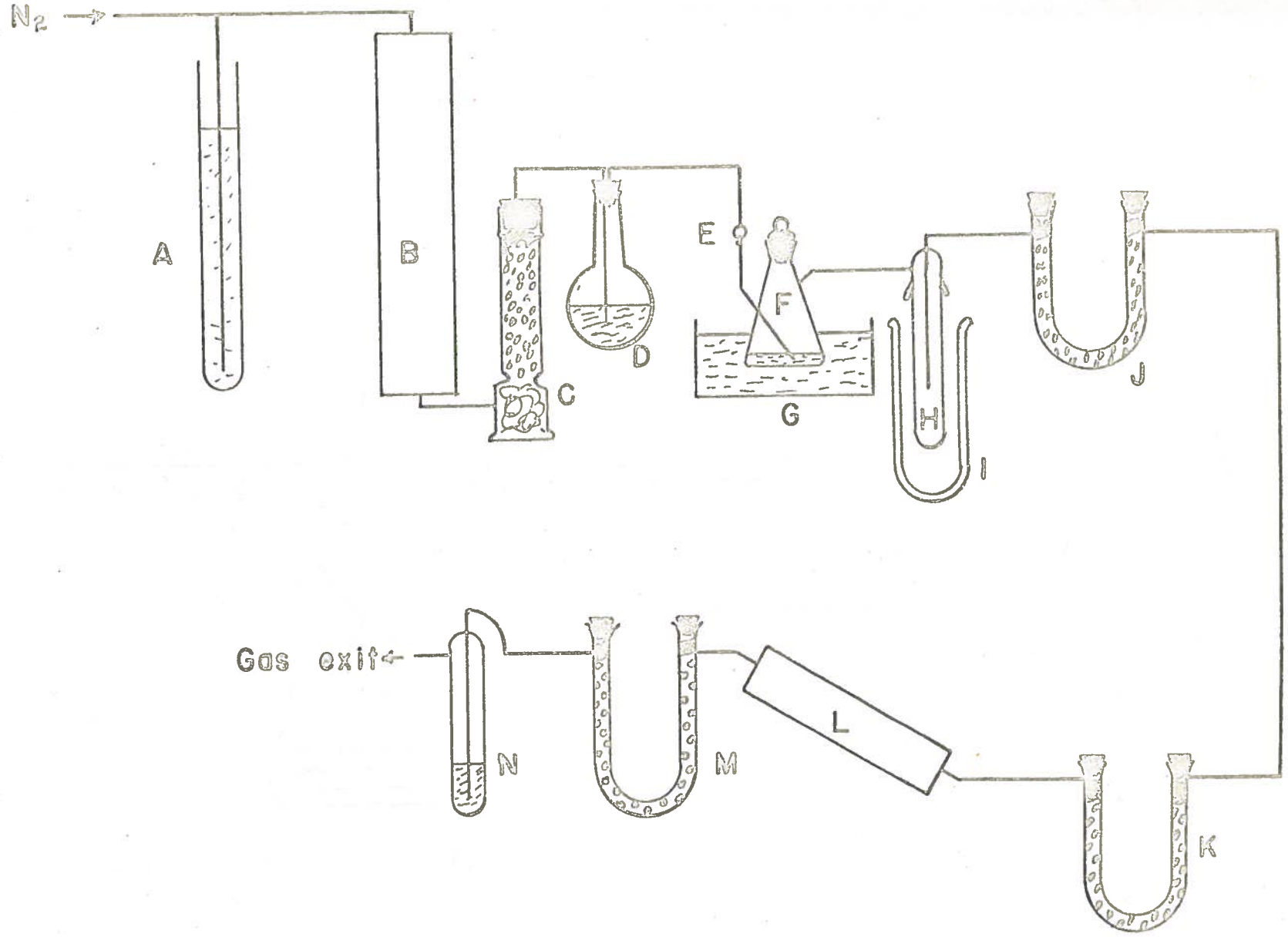


FIGURE 3

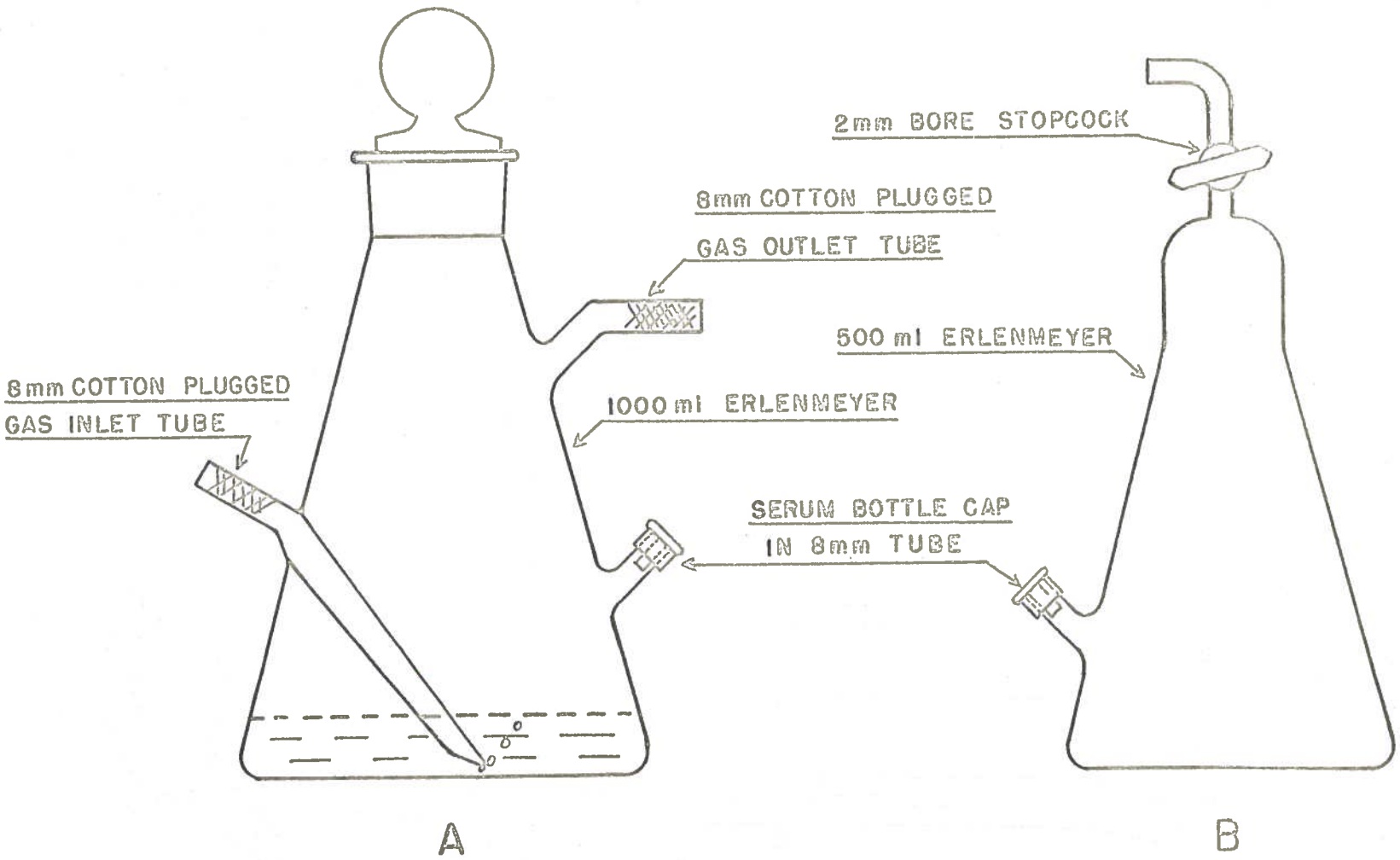

2mm BORE STOPCOCK
8mm COTTON PLUGGED
GAS OUTLET TUBE
500 ml ERLENMEYER
8mm COTTON PLUGGED
GAS INLET TUBE
1000 ml ERLENMEYER
SERUM BOTTLE CAP
IN 8mm TUBE
A
B

Fig 4

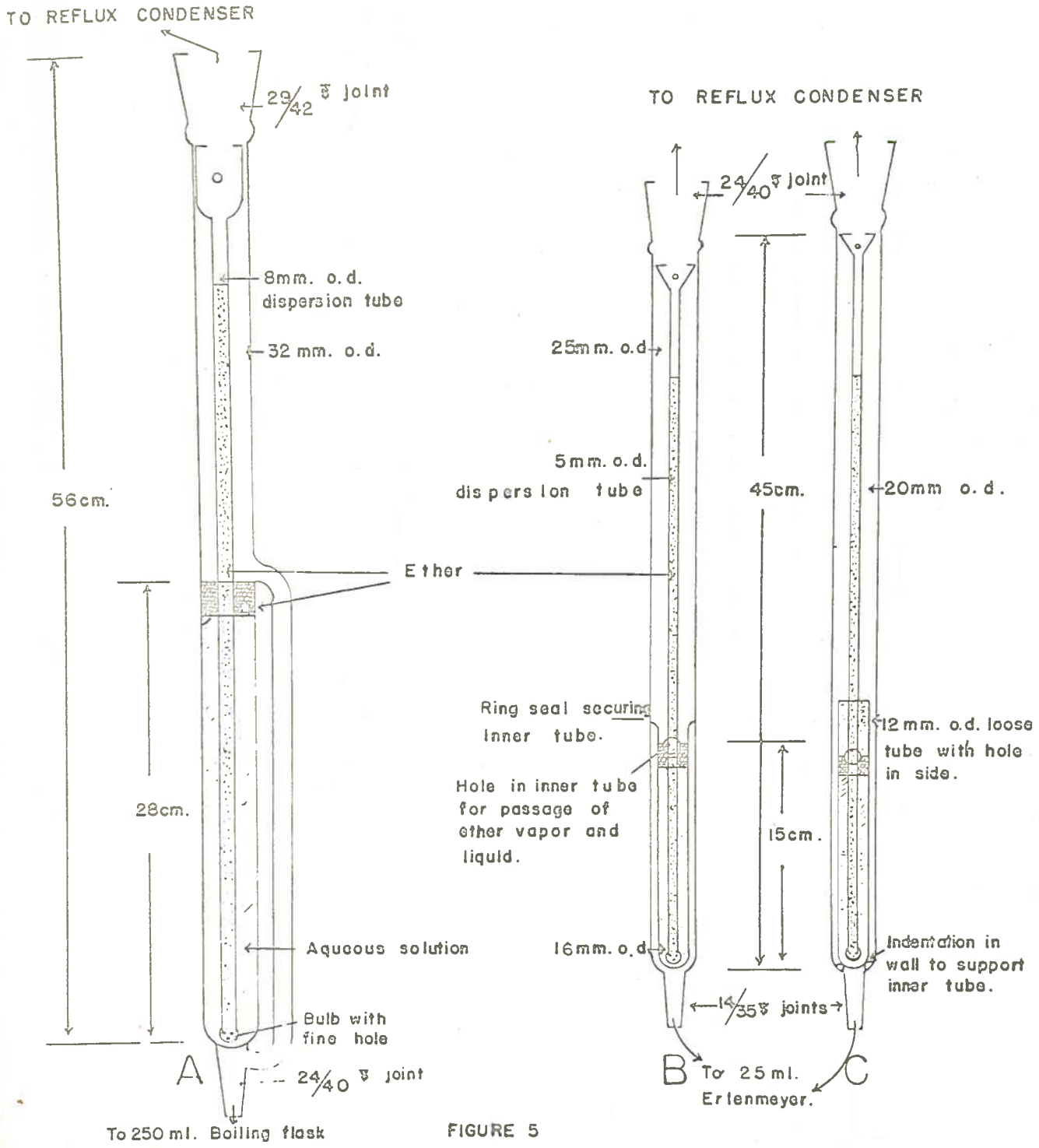

TO REFLUX CONDENSER
29/42 ♂ joint
8mm. o.d.
dispersion tube
32 mm. o.d.
56cm.
Ether
28cm.
Aqueous solution
Bulb with
fine hole
24/40 ♂ joint
A
To 250 ml. Boiling flask
TO REFLUX CONDENSER
24/40 ♂ joint
25mm. o.d.
5mm. o.d.
dispersion tube
45cm.
20mm o.d.
Ring seal securing
inner tube.
Hole in inner tube
for passage of
ether vapor and
liquid.
12 mm. o.d. loose
tube with hole
in side.
15cm.
16mm. o.d.
Indentation in
wall to support
inner tube.
14/35 ♂ joints
B
To 25 ml.
Erlenmeyer.
C

FIGURE 5

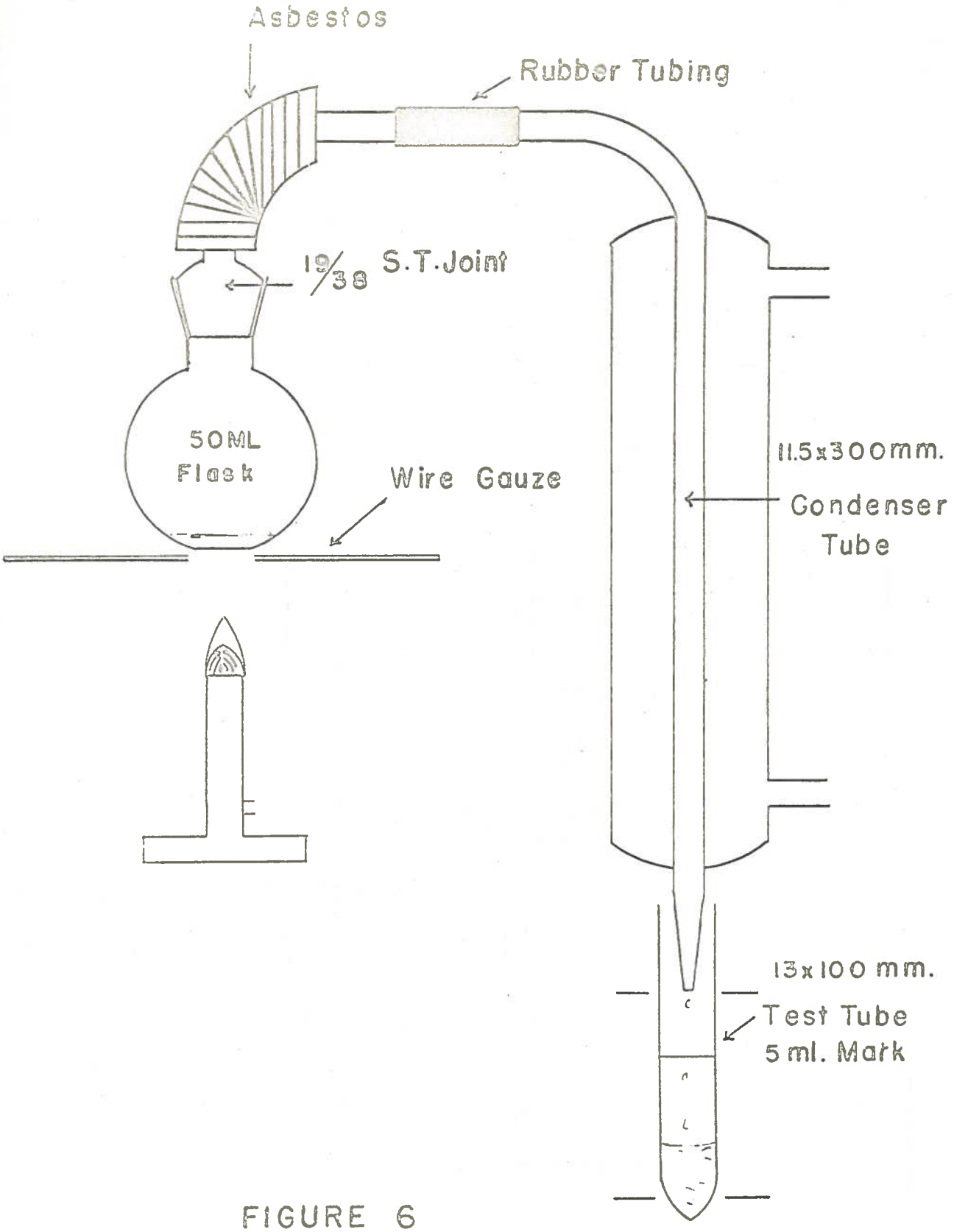

Asbestos
Rubber Tubing
19/38 S.T.Joint
50ML
Flask
Wire Gauze
11.5x300mm.
Condenser
Tube
13x100 mm.
Test Tube
5 ml. Mark

FIGURE 6

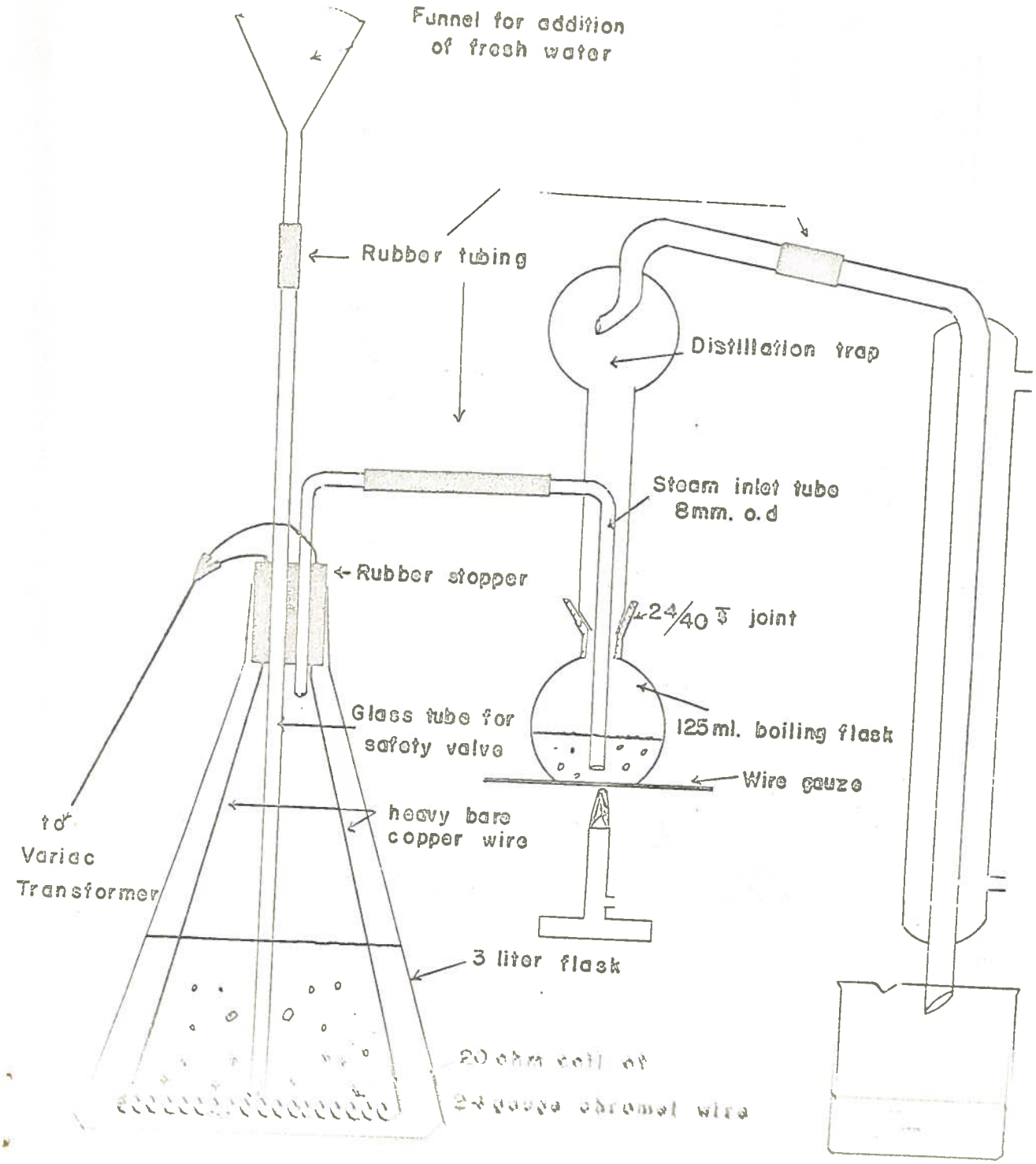

Funnel for addition of fresh water
Rubber tubing
Distillation trap
Steam inlet tube 8mm. o.d
Rubber stopper
24/40 ℧ joint
Glass tube for safety valve
125ml. boiling flask
Wire gauze
to Variac Transformer
heavy bare copper wire
3 liter flask
20 ohm coil of
24 gauge chromel wire

FIGURE 7

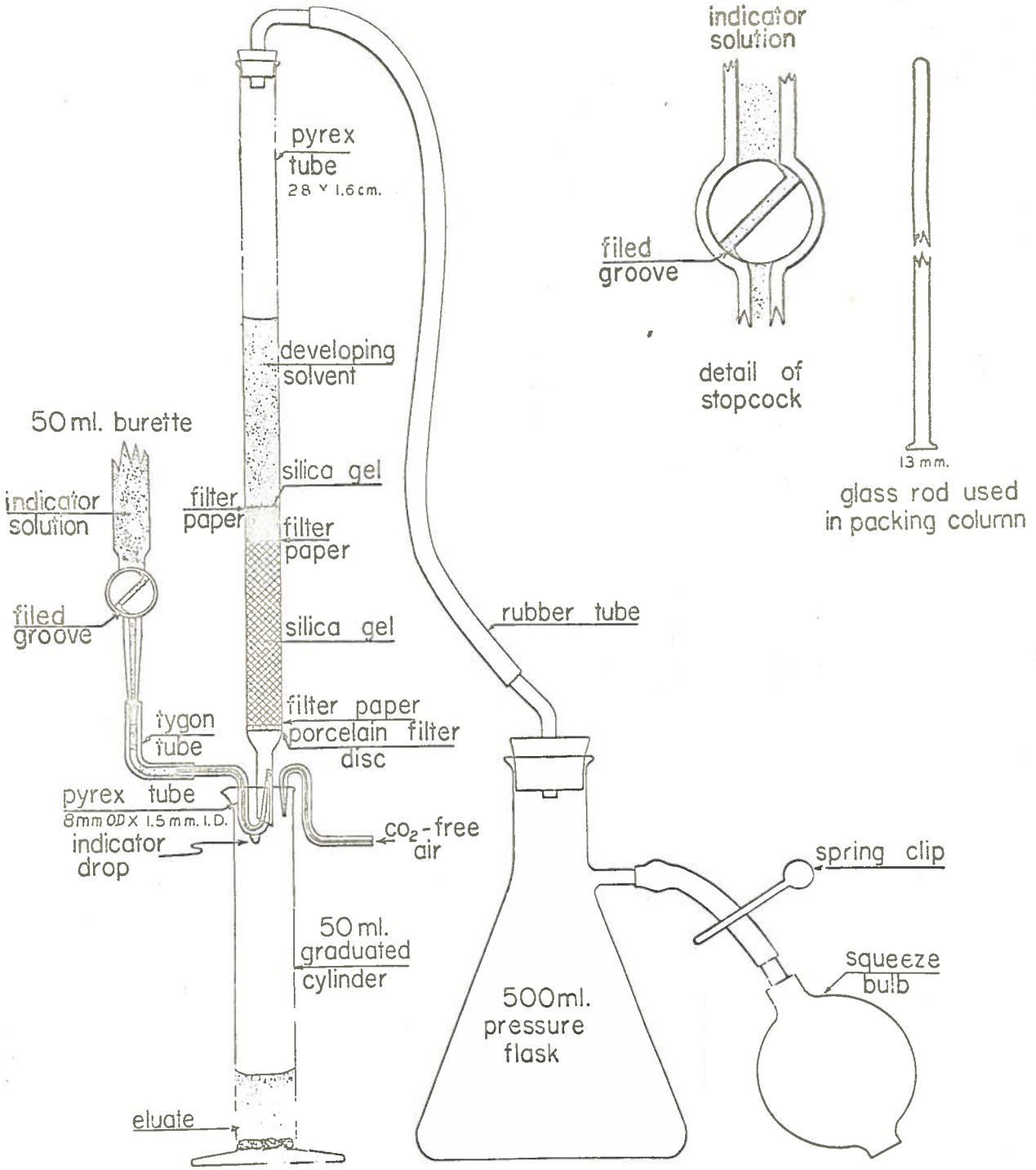

indicator solution
filed groove
detail of stopcock
13 mm.
glass rod used in packing column
pyrex tube
28 × 1.6 cm.
developing solvent
50 ml. burette
silica gel
indicator solution
filter paper
filter paper
filed groove
silica gel
rubber tube
filter paper
porcelain filter disc
tygon tube
pyrex tube
8mm OD × 1.5 mm. I.D.
indicator drop
CO_2-free air
spring clip
50 ml. graduated cylinder
squeeze bulb
500ml. pressure flask
eluate

FIGURE 8

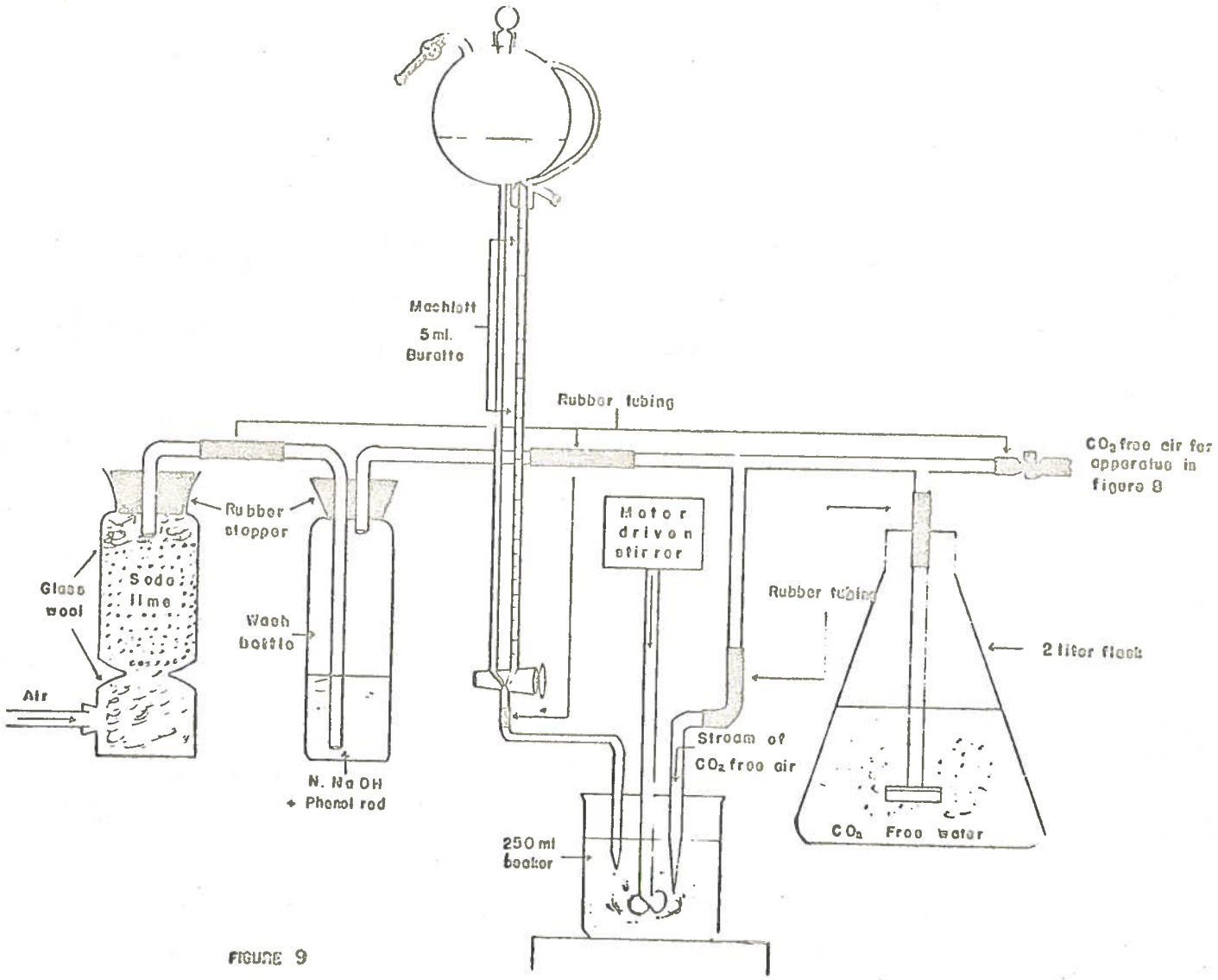

Machlett
5 ml.
Buratte
Rubber tubing
CO_2 free air for apparatus in figure 8
Rubber stopper
Glass wool
Soda lime
Wash bottle
Air
N. Na OH + Phenol red
Motor driven stirrer
Rubber tubing
2 liter flask
Stream of CO_2 free air
250 ml beaker
CO_2 Free water

FIGURE 9

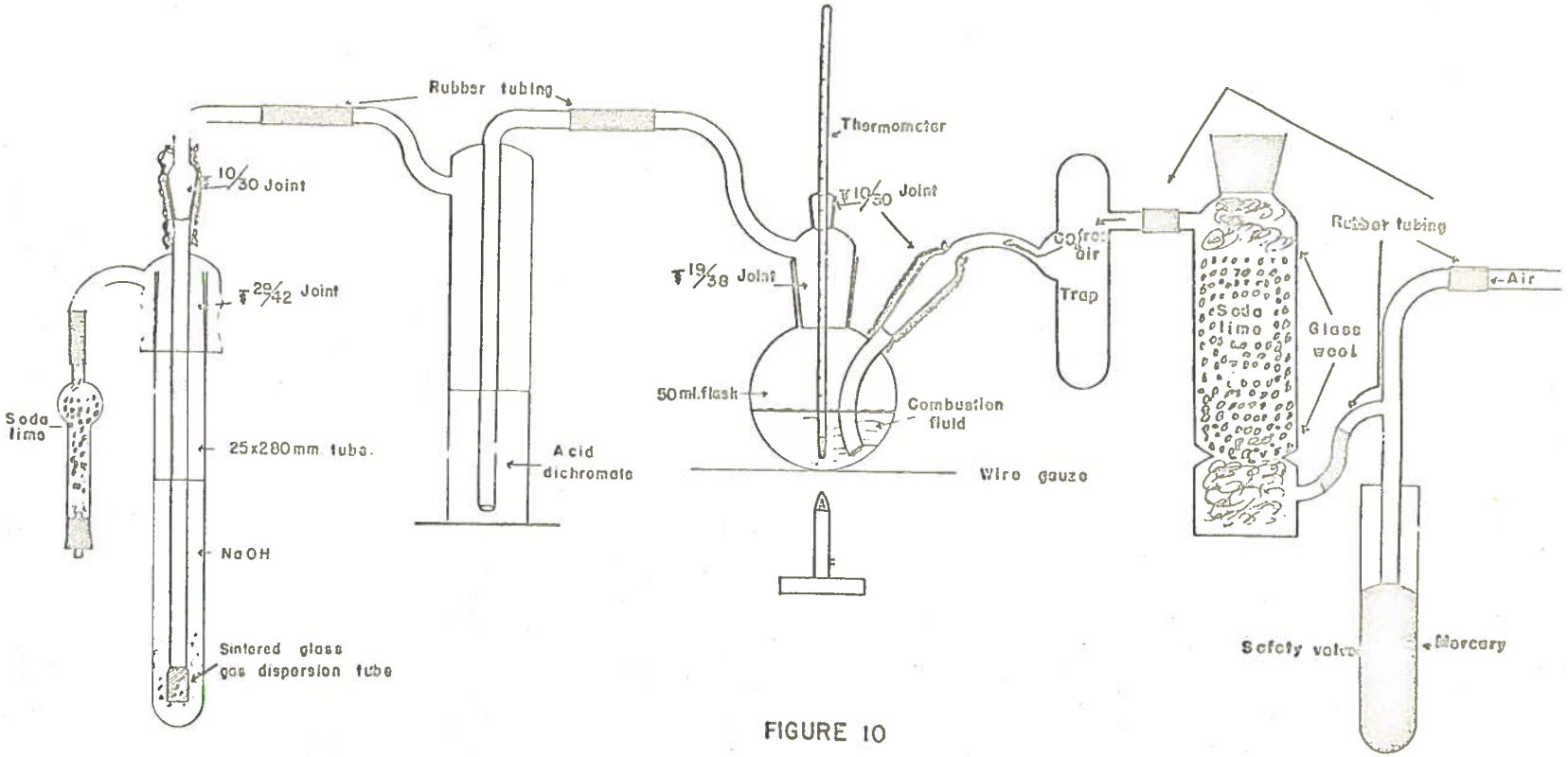

Rubber tubing
Thermometer
10/30 Joint
T 10/30 Joint
T 19/38 Joint
T 29/42 Joint
Soda lime
25x280mm tube
Acid dichromate
50ml.flask
Combustion fluid
Wire gauze
Trap
Soda lime
Glass wool
Rubber tubing
Air
NaOH
Sintered glass gas dispersion tube
Mercury

FIGURE 10

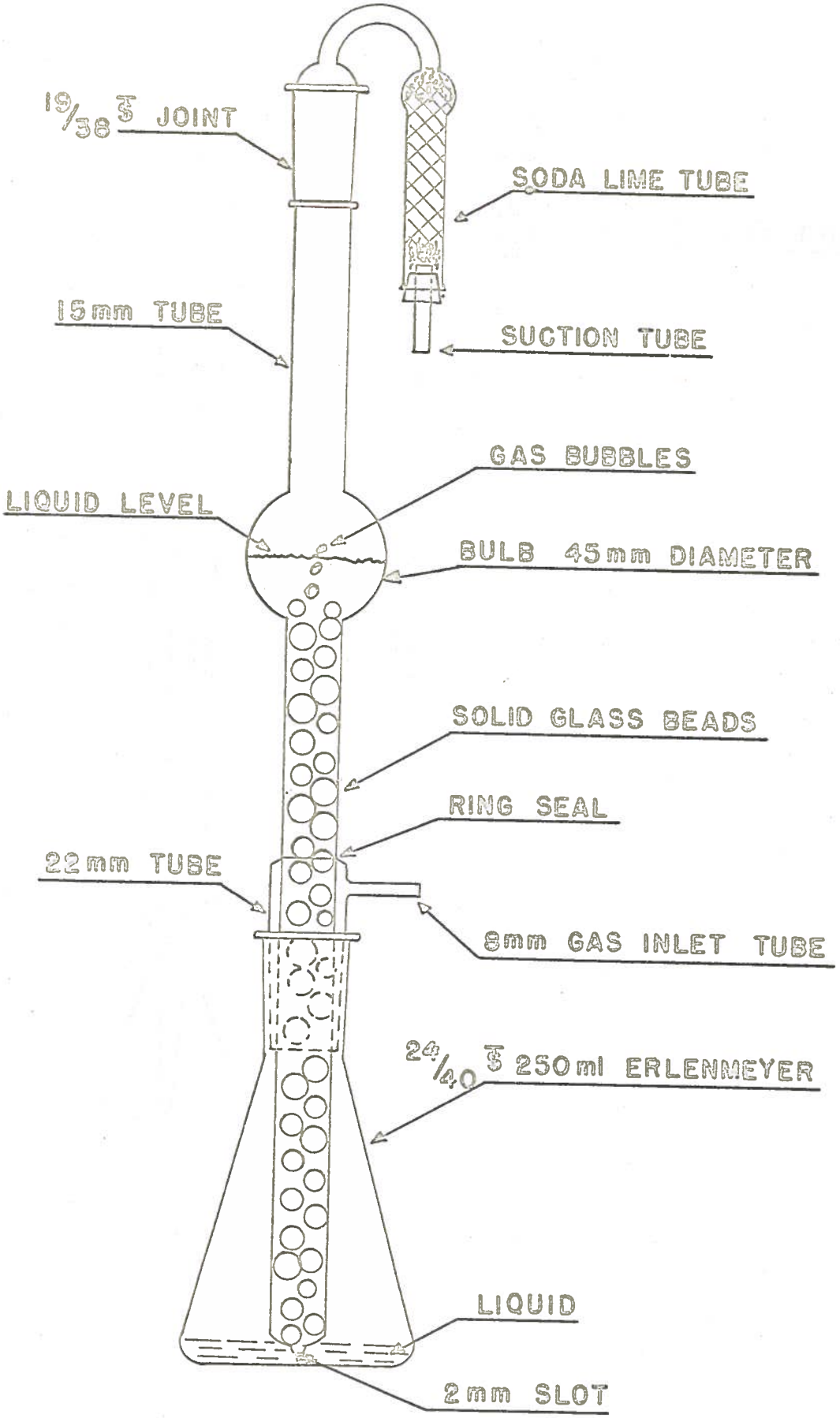

19/38 S JOINT
SODA LIME TUBE
15mm TUBE
SUCTION TUBE
GAS BUBBLES
LIQUID LEVEL
BULB 45mm DIAMETER
SOLID GLASS BEADS
RING SEAL
22mm TUBE
8mm GAS INLET TUBE
24/40 S 250ml ERLENMEYER
LIQUID
2mm SLOT

Fig II

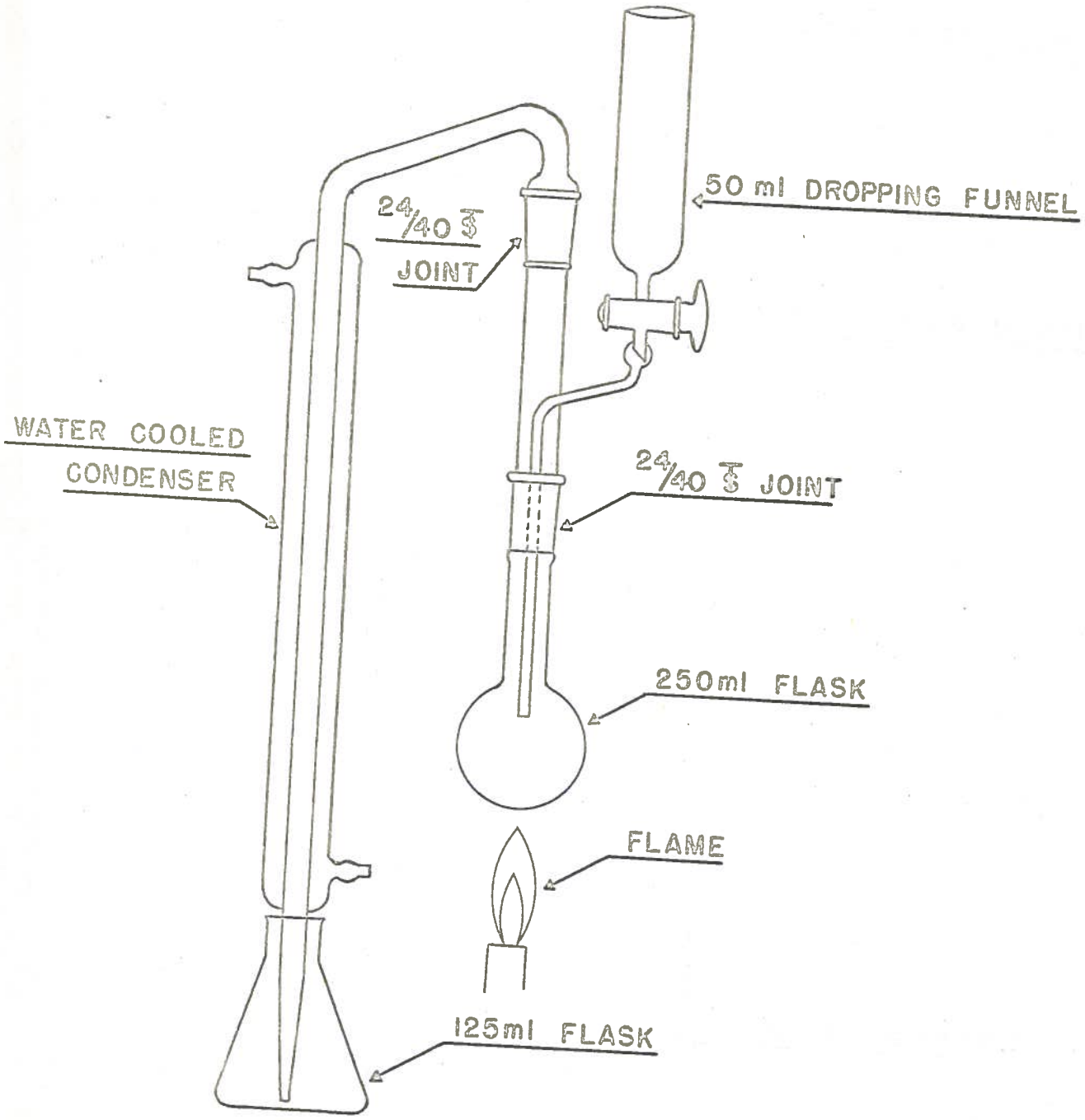

50 ml DROPPING FUNNEL
24/40 ⅄
JOINT
WATER COOLED
CONDENSER
24/40 ⅄ JOINT
250ml FLASK
FLAME
125ml FLASK

Fig 12

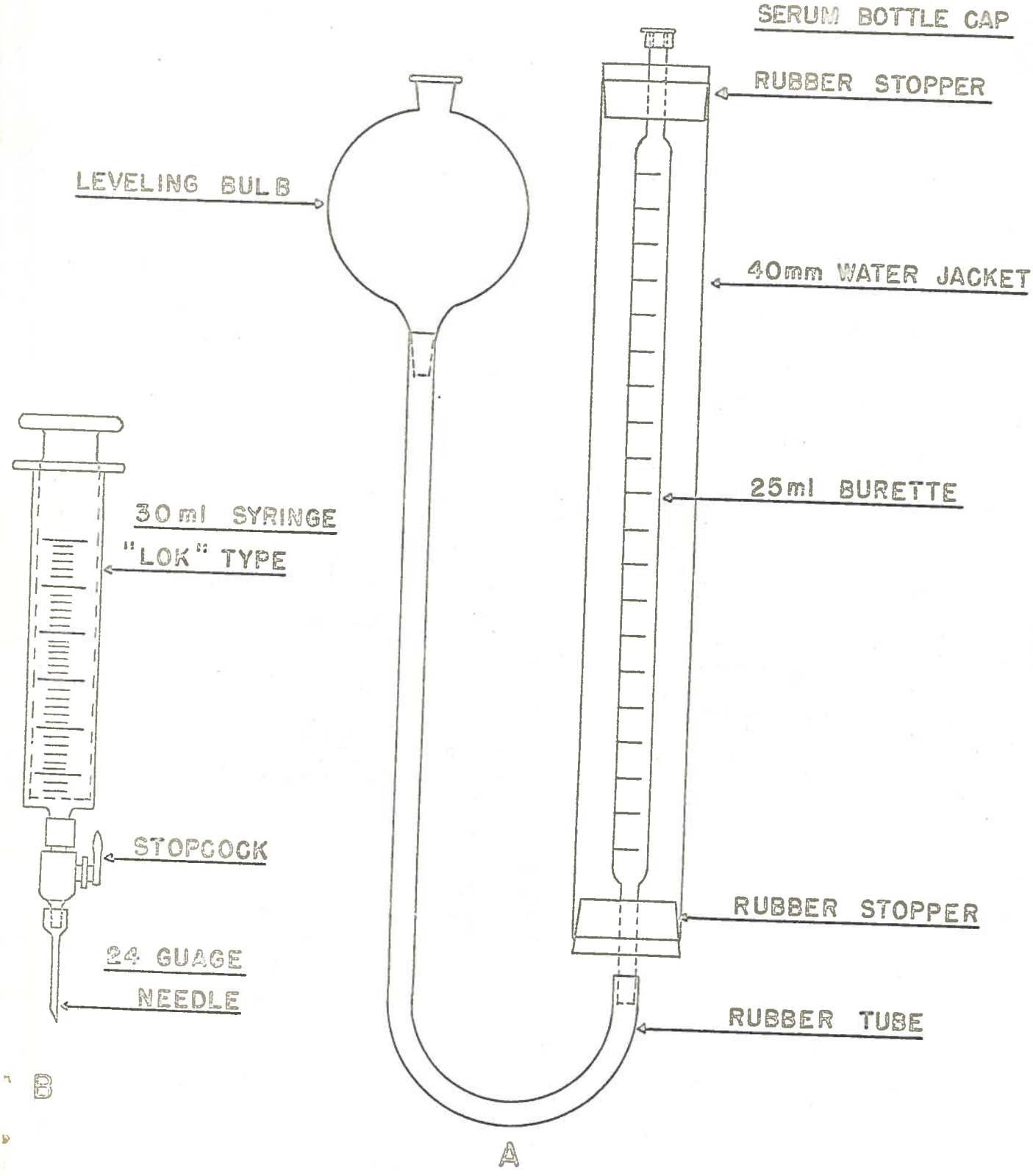

SERUM BOTTLE CAP
RUBBER STOPPER
LEVELING BULB
40mm WATER JACKET
25ml BURETTE
30 ml SYRINGE
"LOK" TYPE
STOPCOCK
RUBBER STOPPER
24 GUAGE
NEEDLE
RUBBER TUBE
B
A

Fig 13

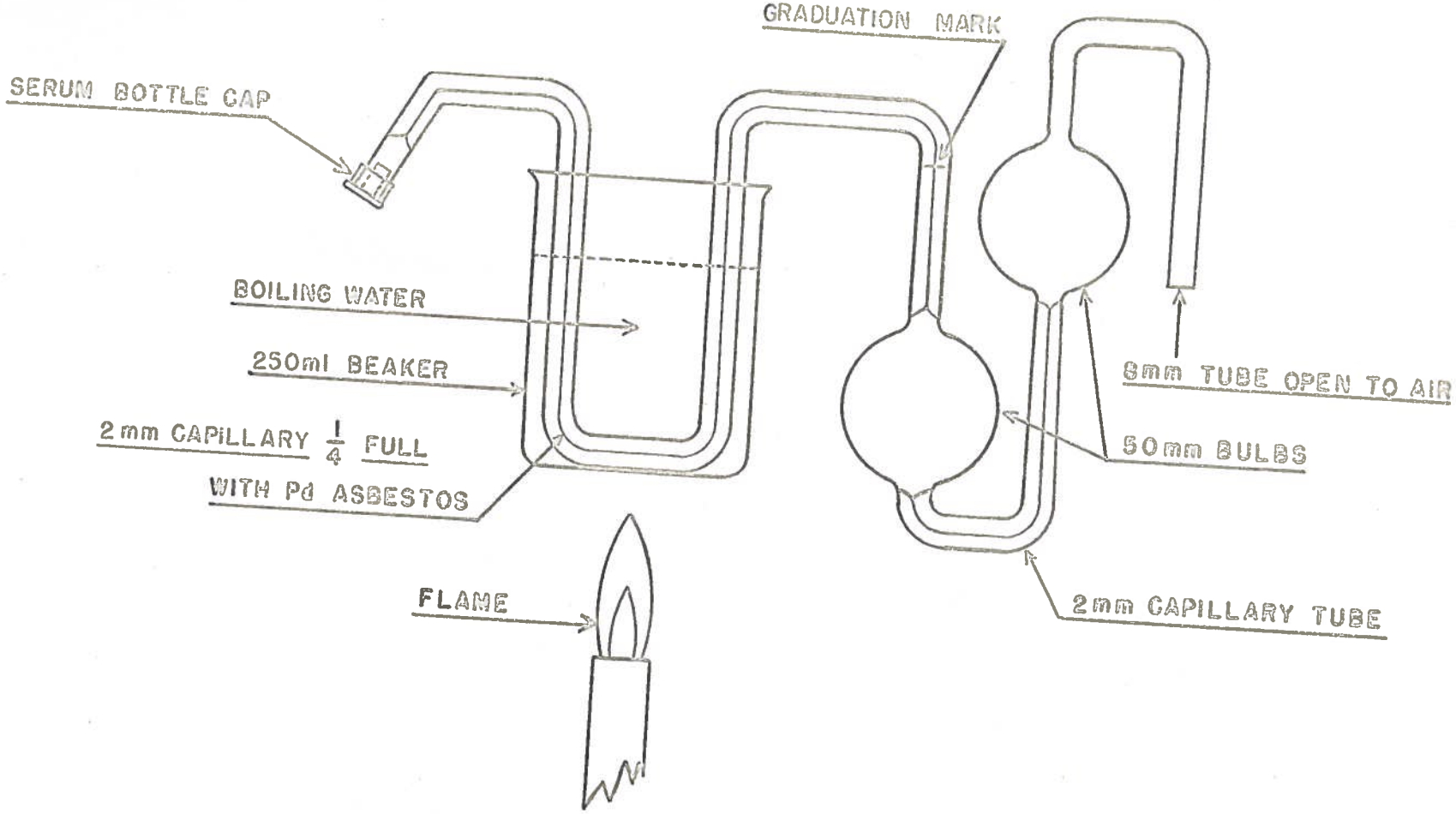

GRADUATION MARK
SERUM BOTTLE CAP
BOILING WATER
250ml BEAKER
2mm CAPILLARY 1/4 FULL
WITH Pd ASBESTOS
FLAME
8mm TUBE OPEN TO AIR
50mm BULBS
2mm CAPILLARY TUBE

Fig 14

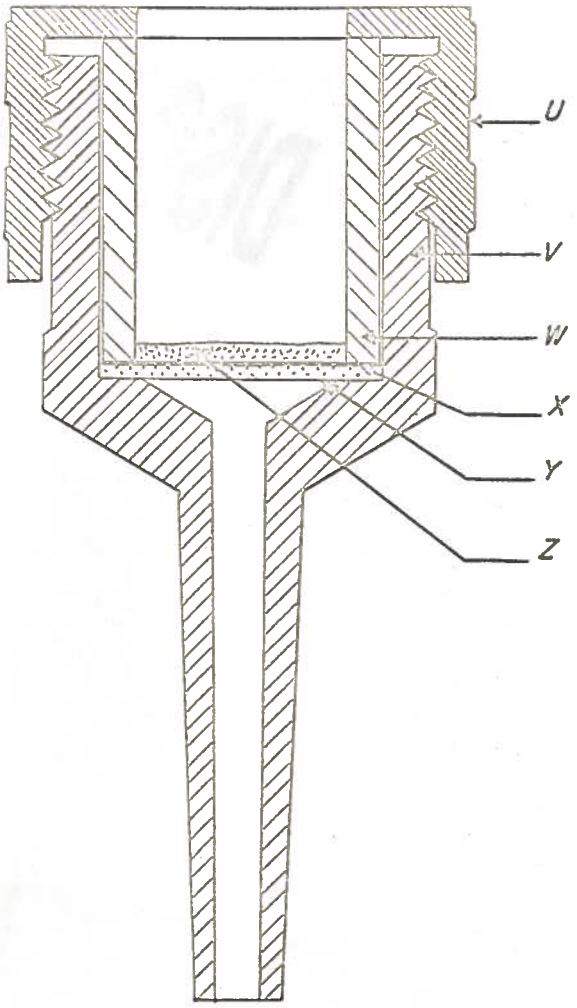


Fig. 15