MANOMETRIC METHODS

By

L. TELEGDY-KOVÁTS

Institute of Food Chemistry, Polytechnic University, Budapest

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As DIXON in his well-known book states, the most convenient methods available for following the reactions in which a gas is either absorbed or evolved are the manometric methods as they are called: *i. e.* methods in which the reaction is caused to take place in a closed vessel attached to some form of gauge-tube containing a liquid by means of which changes in the amount of gas in the vessel can be quantitatively measured. The measurement of the absorption of oxygen or the production of CO_2 by respiring cells, or by oxidation-reduction systems isolated from cells, is fundamental for the elucidation of the mechanism of cell respiration, and manometric methods have been extensively used for the purpose. Their usefulness does not end here: reactions, involving the production or disappearance of acid or alkaline substances, can also be followed manometrically by causing the reaction to take place in a bicarbonate buffer solution in equilibrium with a gas mixture containing CO_2. In this case the production of a given amount of acid will cause a corresponding amount of CO_2 to be discharged which can be read off the manometer. Many hydrolitic reactions, for instance, can be studied in this way.

Manometric methods, adaptable to a variety of purposes, are, in fact, widely and increasingly used in biological laboratories.

The manometers used are of three main types. In the first type the gas in the vessel is kept at constant pressure by adjusting the liquid in a graduated tube connected with it, and the change in volume is read off the tube. The principle involved is that of the Haldane gas analysis apparatus: the Winterstein micro-respirometer is an example of this class. In the second type the vessel is attached to one end of a U-shaped manometer tube, the other end of which freely communicates with the atmosphere. The liquid in the tube is adjusted to keep the gas at constant volume, the change in pressure is read and the amount of gas evolved or absorbed calculated, accordingly. The instrument is commonly called the Warburg manometer on account of its extensive use by Warburg and his school. The third of differential type manometer works neither at

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constant pressure nor at constant volume, both changing simultaneously. In this variety the other end of the manometer tube, instead of freely communicating with the atmosphere as in the Warburg type, is attached to a second vessel similar to the first, designed to eliminate errors due to slight changes of temperature, etc. This is commonly known as the Barcroft type, after the man who developed it in its present form, although the principle had been employed previously by Warburg for another purpose.

There are two ways for making the determinations. In the direct method the CO_2 is absorbed by alkali so that the observed change in the amount of gas in the flask gives the oxygen absorption directly. In the indirect method the respiratory CO_2 is not absorbed but advantage is taken of the fact that the solubilities of oxygen and CO_2 are very different.

Initially, the Warburg technique was only employed in biochemistry, mainly for scientific investigations on the respiration of animals and plants, the assimilation of plants, processes of fermentation, etc. This method soon turned out to be suitable for resolving practical problems of analysis, and questions occurring in industry as well, it furthermore could be utilized in the study of processes not entailing the formation of gases *e. g.* in dilatometry. The versatility of the Warburg method will be demonstrated on practical examples in the following.

The application of yeast species in industry

Like the higher-class plant or animal organisms, yeasts — single-celled — also require energy-producing processes to ensure their biological activity. In general the energy-producing, exergonic reactions taking place in cells and tissues are of oxidative character and can be summarized by the notion of biological oxidation. According to PALLADIN, the respiration of cells and that of tissues — *i. e.* biological oxidation — can take place both in the presence of air (aerobically) and in its absence (anaerobically) *e. g.* in the case of glucose as follows :

anaerobic phase	$C_6H_{12}O_6 + 6\ H_2O + 12\ R \rightarrow 6\ CO_2 + 12\ RH_2$
aerobic phase	$12\ RH_2 + 6\ O_2 \rightarrow 12\ R + 12\ H_2O$
gross equation	$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$

where R is some hydrogen acceptor. Under anaerobic conditions, the hydrogen acceptor is some organic compound ; then the process is denoted as fermentation. Under aerobic conditions, the oxygen in the air constitutes the hydrogen acceptor, this is the process commonly called respiration. The relation of these processes has already been intensively studied by PASTEUR who recognized

that yeast multiplied rapidly parallel with aerobic respiration, and fermentation was repressed in the presence of air, whereas in the absence of air, the process of fermentation was dominant (Pasteur's reaction.).

The utilization of the species of yeasts for industrial purposes depends on their fermenting or respiring ability. That is why investigations conducted in view of the above are of such importance from the viewpoint of practice. For the production of baker's yeast, a species of yeast must be employed wich has a high respiratory activity. The yield in yeast factories is primarily determined by the activity of respiration of the yeasts, that is, by the degree of aeration of the mash. Another requirement to be met by baker's yeast is that it should process a high ability of leavening *i. e.* that it must have an adequate fermenting activity under anaerobic circumstances. With spirit yeasts, respiratory ability only plays a subordinate role since the yeasts are only aerated at the beginning of their production and later — during utilization proper — stress is only laid on energetic, rapid ability of fermentation. Consequently, active and rapid multiplication is disadvantageous for spirit yeasts (this chiefly occurs in the course of respiration) because it entails a reduction in the spirit yield. Identical requirements are to be met by good brewer's yeasts as well. In the production of fodder yeasts — in contradistinction to baker's, spirit and brewer's yeasts — the respiratory activity of the yeasts is of sole importance, no fermenting ability is required at all since the latter would lessen the yield.

The most adequate species and kinds of yeast for the individual branches of industry can be selected on the basis of measurements conducted in the Warburg apparatus. In these tests the volume of oxygen consumed in the course of respiration, the volume of carbon dioxide formed during fermentation in the presence of air and the volume of carbon dioxide formed during fermentation in the absence of air are measured. The usual notations are

$Q_{CO_2}^{N_2}$ = carbon dioxide formed by fermentation in a nitrogen atmosphere, cu. mm

Q_{O_2} = oxygen consumed by respiration, cu. mm

$R_{CO_2}^{O_2}$ = carbon dioxide formed by fermentation in an air atmosphere, cu. mm

Using a Warburg apparatus, PELC has established experimental data (see Table on p. 190) for the respirating and fermenting ability of two baker's yeasts (Saccharomyces cerevisiae I and II), two brewer's yeasts (Saccharomyces carlsbergensis and S. ludwigii) and a fodder yeast (Torula utilis) :

The first column of the Table contains the fermenting ability of the yeasts in a nitrogen atmosphere (*i. e.* free of air), practically conforming to the conditions met with in the leavening of dough or the fermentation of spirit mashes. Listed in the second and third columns are the data on respiration and fermentation

	I $Q_{CO_2}^{N_2}$	II Q_{O_2}	III $Q_{Q_{CO_2}}^{O_2}$	Inhibition of fermentation %
	cu. mm per hour			
Saccharomyces cerevisiae I	274	87	95	66
Saccharomyces cerevisiae II	260	70	150	42
Saccharomyces carlsbergensis	233	8	213	8
Saccharomyces ludwigii	152	45	127	16
Torula utilis	260	180	18	93

simultaneously occurring in the presence of air, during which the nutritive substance is used up partly for respiration and partly in fermentation. The second column shows the volume of oxygen consumed by respiration. The third column shows the volume of carbon dioxide formed by fermentation occurring simultaneously with respiration. In the fourth cloumn, the inhibition of fermentation is expressed percentually in such a manner that the difference between the volumes of carbon dioxide developed in the absence and in the presence of air has been referred to the volume of carbon dioxide formed in the absence of air.

$$\frac{Q_{CO_2}^{N_2} - Q_{CO_2}^{O_2}}{Q_{CO_2}^{N_2}}$$

Namely, total fermentation is characterized by the formation of carbon dioxide in a nitrogen atmosphere whereas reduced fermentation in the presence of oxygen is shown by the data in column III *i. e.* by the volume of carbon dioxide formed in the presence of air.

A thorough analysis of the data proves that the second of the two baker's yeasts is less appropriate for manufacture on account of its lesser respiratory activity and on the fact that fermentation was only reduced by 42 per cent. Conformingly, a lesser yield was produced by this yeast than by the first, because the higher fermenting ability reduces yield. Of the two investigated brewer's yeasts, S. carlsbergensis is positively the better since it has a low respiratory activity in the presence of oxygen next to a relatively favourable fermenting ability and the latter is, therefore, but very slightly reduced. S. ludwigii respires in a lively manner and this property is not propitious from the viewpoint of brewing, active fermenting ability being decisive. Finally, the species Torula utilis, used for fodder yeast, possesses a fermenting activity in oxygen-free media, but its respiration in the presence of oxygen being unusually active, fermentation ceases almost completely, the degree of fermentation inhibition attains 93 per cent. Thus, Torula utilis can be used to best advantage for multiplication by aeration since the almost complete lack of fermentation ensures a good yield.

Other investigations based on the metabolism of microorganisms

The respiration of fermenting ability of microorganisms can be influenced by the addition of various chemicals. The efficiency of the additive can be checked by the use of a Warburg apparatus to establish the degree of influencing. Flour improving substances *e. g.* $KBrO_3$ can be checked by their effect on baker's yeast, detergents such as quaternary ammonium compounds, by their bactericidal action on bacterial cultures as described by BENIGNO and BERTI. and preserving agents by their bactericidal or bacteriostatic action on various microorganisms according to KIERMEIER. Conclusions may be drawn from the slope of the straight line plotted on the basis of the oxygen consumption or carbon dioxide formation observed in the Warburg apparatus and the duration of the test.

Another very important field of application of the Warburg technique is the direct determination of the biochemical oxygen requirements of sewage. Formerly, the sewage was first diluted with water for the determination. However, since the quality of the diluent bears a considerable influence on results in the indirect process, a direct method has been elaborated by JAEGERS and NIEMITZ making use of the Warburg apparatus. An advantage of this method is that subsequent to the determination of oxygen absorption, further tests can be carried out on the sewage *e. g.* investigation whereby impurities and their oxidation products may be established. 125 ml Erlenmeyer flasks should be used in these tests instead of the usual small Warburg vessels and the carbon dioxide formed must be absorbed in a cuvette containing an alkaline solution.

Enzymatic analysis

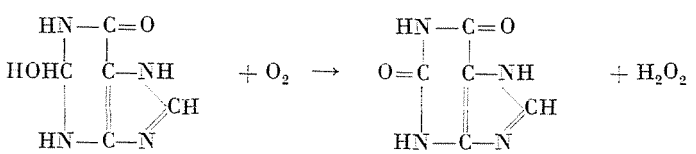
Enzymatic analysis comprises those up-to-date methods in which pure enzyme preparations are used. In contrast to modern microbiological analyses where the enzymes are bound to the living cells, enzymes isolated from the living cells or tissues are employed in enzymatic analysis. The important role of enzymes in the determination of certain constituents of natural organic substances is due to their particular properties. This was recognized in the initial stage of the development of enzyme chemistry. Enzymes possess two properties, advantageous from the viewpoint of utilization in analysis. These are the specificity of their catalytic action and their high degree of sensitivity towards chemical and physical influences. Based on these properties, the procedures of enzymatic analysis may be classified into two groups. To the first group belong „substrate-specific" enzymatic methods based in theory on exposing the substance to be tested to the action of an enzyme specific towards the constituent to be determined and then establishing the products of enzymatic

degradation. The second group comprises procedures in which enzyme activity is either increased or reduced by the substance tested, and the quantity of the influencing substance is inferred from the changes in enzyme activity. Since these enzyme inhibitors and activators may be comprehensively denoted as „effectors" according to BERSIN, procedures classified in the second group may be summarized under the denomination of „enzymatic effector analyses". Manometric determinations are also relied upon in the execution of methods belonging to either group. Possibilities for applying the Warburg apparatus for such purposes will be demonstrated on a few examples in the following.

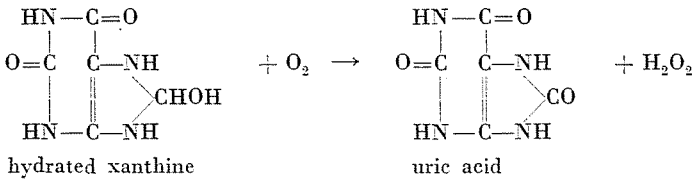
Substrate-specific methods

A considerable advantage of procedures classified under this group over purely chemical methods is that the required component can be determined without having to separate the accompanying substances previously. Only substrates of enzyme action can, evidently, be determined in this manner. Since most natural organic substances are enzyme substrates, the greatest number of biologically important compounds may be determined in this way. An indispensable condition of successful work are carefully purified enzyme preparations since specificity decreases in parallel with the degree of contamination. The greater part of unfavourable experiences gained in enzymatic analyses can be traced to the employment of inadequately purified enzyme preparations.

Typical of the substrate-specific procedures carried out with the use of Warburg apparatuses are those for which yellow oxidation enzymes are applied. Yellow oxidation enzymes belong to that group of oxydation enzymes which contain riboflavin (vitamin B_2) as a coenzyme. The specificity of these enzymes depends on the structure of the protein moiety (apoenzyme). Three enzymes of this group are the most often used in enzymatic analyses, xanthine oxidase (Schardinger's enzyme), glucose oxidase and D-amino acid oxidase. Schardinger's enzyme — produced from liver and kidney — is employed for determining hypoxanthine and xanthine which are oxidized into uric acid by enzymatic action. The reactions are as follows:



hydrated hypoxanthine xanthine



hydrated xanthine uric acid

Dehydrogenation actually takes place, the liberated hydrogen is bound to the molecular oxygen in the air. H_2O_2 is formed temporarily which, having a deleterious influence on enzymatic action, must be broken down with catalase. Although the specificity of the enzyme is relatively low because it also oxidizes adenine and certain aldehydes besides xanthine and hypoxanthine, a procedure has been developed by KREBS and GERSTRÖM for the quantitative determination of both oxypurine derivatives in quantities of 0,1 mg. This procedure determines not only the uptake of oxygen but also the quantity of uric acid formed. Oxidation of the aldehydes does not involve uric acid formation and the oxidation of adenine takes place at a very low rate. Xanthine and hypoxanthine could thus be determined simultaneously. The oxygen consumed is determined with a Warburg apparatus and the uric acid formed is established colorimetrically in the course of the procedure. Let x_{O_2} = mol O_2 used up in the reaction and x_u = mol uric acid formed in the reaction, then — taking into account that on the basis of the above reaction equations — 1 mol O_2 is required to oxidize hypoxanthine to uric acid and ½ mol O_2 is needed for the oxidation of xanthine

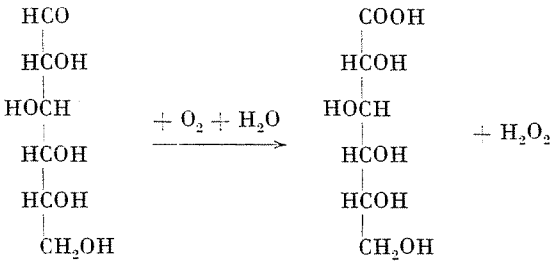
$$\text{hypoxanthine} = 2\,x_{O_2} - x_u$$

$$\text{xanthine} = 2\,(x_u - x_{O_2})$$

Both hypoxanthine and xanthine being important components of meat and meat extracts, the determination is of eminent importance from the viewpoint of biochemistry and food analysis.

Glucose oxidase (notatin) occurs in fungi of low order; D-glucose is oxidized into gluconic acid by its action, oxygen in the air acting as a hydrogen acceptor. The reaction is the following:

$$C_6H_{12}O_6 + \tfrac{1}{2}\,O_2 = C_6H_{12}O_7$$



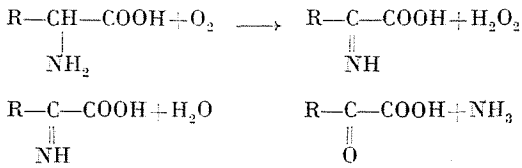
The temporarily formed H_2O_2 is broken down with catalase. If the reaction takes place in the presence of alcohols, the latter are converted into aldehydes by the action of catalase. The following reaction takes place in this case:

$$C_6H_{12}O_6 + O_2 + R \cdot CH_2OH = C_6H_{12}O_7 + H_2O + R \cdot CHO$$

In the presence of excessive amounts of alcohol, oxygen consumption is the double of that observed in the oxidation of glucose.

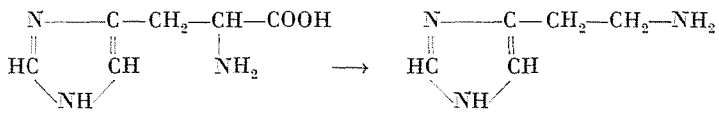
The specificity of the enzyme is satisfactory according to KEILIN and HARTREE. The method is not only adequate for the determination of free glucose but also for the study of such enzyme reactions in the course of which glucose is liberated. In this manner the hydrolysis of sucrose by β-h-fructosidase, that of maltose by maltase and the hydrolysis of starch by amylase, the decomposition of glucose phosphates by phosphatase and the cleavage of glucosides by glucosidases may be readily followed. The used-up oxygen is determined in each case in a Warburg apparatus.

The presence of D-amino acid oxidase can be detected in most animal tissues, it is produced from kidneys and livers. By its action keto acids are formed from amino acids of non-natural D-configuration by the oxidative splitting-off of the amino group. The course of the reaction is



The enzyme has a very specific action and, therefore, is indispensable for the identification of any D-amino acids present in natural L-amino acid mixtures. It, furthermore, can be utilized for the identification of D-peptidases according to studies by HERKEN and ERXLEBEN. The oxygen used up in the reaction is determined in a Warburg apparatus.

From among the substrate-specific procedures of enzymatic analyses, the determination of amino acids by amino acid decarboxylases has attained special significance recently. These enzymes, extracted more or less easily from bacteria their coenzyme being pyridoxal-5-phosphate, convert L-α-amino acids into amines in a very specific manner. From histidine *e. g.* histamine is formed according to the following reaction equation:



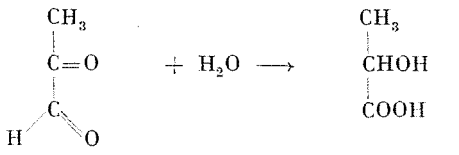
histidine histamine

The method can be applied to best advantage to the analysis of protein hydrolysates since enzymes specific to various amino acids can be extracted from various bacteria. In all cases, the technique of determination is based on the manometric determination of CO_2 liberated in the course of the reaction. This is carried out in a Warburg apparatus. Determination of essential amino acids in this manner is of special interest from the viewpoint of food chemistry.

Enzymatic effector analyses

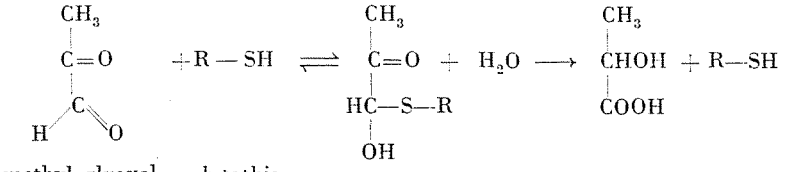
Enzyme-analytical methods belonging to this group are rather low in number the cause of which is that an exact knowledge of the kinetics of the process is required for the analytical evaluation of the action of the effector. Evaluation will only be successful if the influence of the various effectors acting simultaneously can be separated. The procedure itself consists of measuring enzyme activity, first, in the presence of the effector and, second, without addition of the effector. Conclusions in respect to the quantity of the effector may be drawn from the changes in activity. Since enzymes, as catalysts, are very sensitive to effectors, the sensitivity of the determination is of the same degree, that is, such small quantities of effectors can be revealed by the method which, otherwise, could only be established by spectral analysis.

A good example for these procedures is the determination of glutathione with glyoxalase. Glyoxalase, which can be produced from yeast, liver, kidneys, muscles, the seeds of plants of higher order and of bacteria, converts methyl glyoxal into lactic acid according to the following equation :



methyl glyoxal lactic acid

The action of the enzyme requires the presence of glutathione, very small quantities of which considerably increase enzyme activity. According to JOWETT and QUASTEL, methyl glyoxal first combines with glutathione to a transitory product, then glutathione is again set free under the formation of lactic acid.



methyl glyoxal glutathione lactic acid

The reaction is specific to such an extent that it may be employed for the determination of very small quantities of glutathione. According to ENNOR, determination is effected in a Warburg apparatus by measuring the carbon dioxide liberated from the added bicarbonate buffer. The amount of carbon dioxide is proportional to lactic acid formation. The unknown glutathione concentration of a substance may be determined by means of a curve plotted on the basis of tests conducted with the addition of known quantities of glutathione.

Dilatometric procedures

Besides studies of biological gas exchange and procedures of enzymatic analysis, Warburg apparatuses can be used for other purposes such as the carrying out of physical measurements. An interesting field of application are dilatometric measurements in Warburg apparatus on substances whose investigation does not yield satisfactory results when conducted in classical-type dilatometers. Taking into account the findings of BAILEY, according to which the regular motion of measuring liquids (mercury, water, etc.) used in classical dilatometers is disturbed by the voids always occurring inside fats, GIDDEY and EGLI have recently employed a Warburg apparatus for studying the crystalline polymorphism of cocoa butter and that of fats in general. The Warburg apparatus may be used to control dilatometric changes under circumstances which, otherwise, would cause dificulties. Such circumstances *e. g.* are rapid cooling, crystallization at various temperatures, the effect of inoculations effected with microcrystals of different modification, etc. Such dilatometric tests have proved very suitable for the study of the crystalline state of triglycerides and have contributed much to the elaboration of a scientific technique for the manufacture of chocolate and to the knowledge of the causes of recrystallization causing the grey co'our (fat bloom) of chocolate.

Warburg apparatuses may be used for dilatometric measurements without any alteration, pure paraffin oil must only be employed for a sealing liquid instead of the Brodie solution. Furthermore, the apparatus is filled with dry nitrogen gas in order to eliminate the faults caused by water vapour changing its tension in conformity with the pressure. Knowledge of the initial volume of the apparatus and of the volume established at the end of the test are required for the determinations *i. e.* for the calculation of the changes in volume. Conforming to the laws of gases, let

V_0, P_0, T_0 be standard parameters,

V_1, P_1, T_1 be parameters at the beginning of the measurements and

V_2, P_2, T_2 be parameters at the end of the tests, then

$$\frac{P_0 V_0}{T_0} = \frac{P_1 V_1}{T_1} = \frac{P_2 V_2}{T_2}$$

from which

$$V_1 = \frac{P_2 V_2 T_1}{T_2 P_1}$$

Computation of the initial volume thus becomes easy since the final volume [V_2 = volume of the apparatus — volume of the liquid fat] at test temperature (T_2) is known together with the pressure prevailing at the end of the test [$P_2 = P_1 + h$], where P_1 is the pressure at the beginning of the measurement, h being the difference of height read on the manometer. Pressures are expressed in millimeters of the sealing liquid. The specific gravity of paraffin oil (d = = 0,8712 at 21° C) should be taken into account in the calculations.

The modifications of glyceride crystals occurring in cocoa butter specimens variously crystallized can be determined by this method, for example. The dilatation curve of the specimen containing the metastable crystal modification causing grey colour (fat bloom) does not change uniformly.

Summary

Initially, manometric methods have been only employed in biochemistry, mainly for scientific investigations on the respiration of animals and plants, the assimilation of plants, processes of fermentation etc. These methods soon turned out to be suitable for resolving practical problems of analysis, and questions occurring in industry as well, they furthermore can be utilized in the study of processes not entailing the formation of gases *e. g.* in dilatometry. The versatility of manometric methods are demonstrated on practical examples.

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Professor L. TELEGDY-KOVÁTS, Budapest XI., Budafoki út 4—6, Hungary.