A MICRO-METHOD FOR THE DETERMINATION OF ENZYME ACTIVITY.

By B. N. Sastri and M. Sreenivasaya.

The detection and characterisation of enzymes in small quantities of material, and accurate measurements of their activity, are problems which constantly confront the bio-chemist. A micro-technique applicable to the minute quantities of tissue-fluid or sap extractable from the living cell presents special advantages in such investigations.

Principle of the method.—During enzyme action the substrate undergoes change in its molecular state and, therefore, in some of its physical properties. Attempts have been made from time to time to follow the activity by determining changes in viscosity (Spriggs, *Z. physiol. Chem.*, 1902, **35**, 465–494), electrical conductivity (Sjoqvist *Skand. Arch. Physiol.*, 1895, **5**, 277–375; Oker-Blom, *Skand. Arch. Physiol.*, 1902, **13**, 359–374; Henri and Bayliss, *J. Physiol.*, 1907, **36**, 221; Euler, *Z. physiol. Chem.*, 1907, **51**, 213), optical activity, (Schutz, *Z. physiol. Chem.*, 1885, **9**, 577; Schutz and Huppert, *Pfluger's Archiv*, 1900, **80**, 470), refractive index (Obermeyer and Pick, *Hofm. Beitr.*, 1905, **7**, 331), freezing-point (Oker-Blom, *Skand. Arch. Physiol.*, 1902, **13**, 359–374) and dilatation (Van t' Hoff, *Sitz.-Ber. K. Preuss. Akad.*, 1910, **48**, 965). The method now suggested takes advantage of the change of vapour-pressure due to the increase in the number of molecules which results from enzyme-hydrolysis, and is an application of Barger's well-known microscopic technique (*J.C.S.*, 1904, **85**, 286) for determining molecular weights.

Yamakami (*Biochem. J.*, 1920, **14**, 103 and 522) in the course of his studies on caseinogen, has subjected Barger's technique to rigorous tests and made a study of such disturbing factors as have to be taken into account. The present study indicates its suitability for the determination of enzyme activity.

At the outset, it is pertinent to point out that the fundamental difference between the solutions of Barger and Yamakami on the one hand and ours on the other, is that the former consist of 'static' solutions while ours comprise 'dynamic' mixtures, continuous changes in their molecular state being involved.

The vapour-pressure of a solution depends upon its molecular concentration and any alteration in the latter will, according to Wullner's law, involve a change in the vapour-pressure, provided the dissolved substance is non-volatile. If in a closed space there are two vessels, one of which contains the substrate solution alone and the other the substrate solution to which an active enzyme has been added, an isothermal distillation of vapour from the former to the 'active' solution takes place in virtue of the change in molecular concentration

in the latter. This kinetic principle has been applied in following enzymic activity, employing capillary tubes to hold the drops of 'active' and 'standard' solutions. In Barger's words "each drop is placed between two others of a different solution and can evaporate on either side into a small closed air chamber. The chamber is soon saturated with the vapour which can condense freely on the drops. If the vapour-pressures of the two solutions are equal the evaporation will equal the condensation and there will be no change in the volume of the drops. If, on the other hand, the vapour-pressures are unequal there will be a gradient of vapour-pressure in the air spaces. Some drops will therefore be in an atmosphere the vapour-pressure of which is greater than their own. Condensation will take place on these drops and they will increase. The others alternating with these will have a vapour-pressure greater than that of the adjoining air spaces; these drops will evaporate and thus decrease. Thus there is a distillation from the drops of one series to those of the other series, although all are of the same temperature." Hence if a series of drops consisting of the 'active' solutions be alternated with a series of inactivated controls in a thin sealed capillary tube, the change in the size of the 'active' drops, which increase at the expense of the 'controls', represents a measure of the change in the number of molecules and hence of the enzyme action.

PRELIMINARY OPERATIONS.

Capillary tubes.—Short pieces of previously cleaned soft glass tubing (½-inch bore) were drawn out into sections 1–2 feet long and having a bore of 1–2 mm. About 8 cms. in the middle, representing the region of uniform diameter was cut off and preserved in a test tube until use.

'Experimental' and 'Control' solutions.—The experimental or active solutions are those of the substrate to which a known quantity of active enzyme has been added. The control or standard solutions are those of the substrate containing the same amount of enzyme which has been inactivated by boiling for 15 minutes. For the work on diastase, the substrate consisted of a sterilised 2 per cent. solution of soluble starch; the amount of taka-diastase employed was 100 mg. per 100 c.c. of solution. For the work on invertase a 10 per cent. solution of cane-sugar was employed and 5 c.c. of the enzyme preparation added to 300 c.c. of the sugar solution. The enzyme was prepared by Hudson's method (*J. Amer. Chem. Soc.*, 1908, **30**, 1567) from autolysed yeast-liquor.

Filling the tubes.—This requires practice. The smooth-edged tube is taken between the middle finger and the thumb, and by careful manipulation of the index finger alternate drops of the control and active solutions are drawn in. The first and the last drops, both consisting of the control solutions are made about 5 mm. long, the inter-

mediate drops being about 0·5 mm. The terminal drops are shaken down to about 1 cm. from the ends of the tube which is then sealed. The first and the last drops are not measured for they generally decrease by evaporation into the air spaces beyond them and are also most liable to be heated while sealing the tubes. The distance between neighbouring drops is about 4 mm.

Measurement with the microscope.—A series of four tubes was usually prepared at one time and, for convenience in working with the microscope, attached to a slide. The latter was then immediately transferred to a small water-bath, $3 \times 2 \times \frac{1}{2}$ inches, placed on the microscope stage. In this way a fairly uniform temperature (actual variation $\pm$ 0·5°) was ensured. A sharp image of the drop revealed the meniscus as very distinct, and the distance between the two, which gives the minimum thickness of the drop, could be measured. The exact coincidence of the meniscus with the zero of the scale is established before taking the readings. The magnification used was such that one division on the micrometer-drum usually corresponded to 1·06μ (in some experiments 0·625μ), a Watson micrometer eye-piece being used. The results are expressed in hundredths of a scale division.

EXPERIMENTAL OBSERVATIONS.

For the work on diastase a sample of taka-diastase obtained from Parke Davis & Co. was employed. The amount of maltase in the sample was negligible and it was assumed that the enzyme converted the starch entirely into maltose. The reducing sugars were therefore always estimated as maltose. Using only one drop of the changing or active solution between two drops of inactivated control solution, the distance between the two drops being about 0·5 cm., micrometer readings were taken at regular intervals of one hour. At noted intervals also the maltose present in a large-scale experiment, conducted with the same reaction mixture, was estimated by Bertrand's method. The readings obtained were as follows:—

TABLE I.

Action of taka-diastase on starch at 24°.

A.—Micro-Method.

Duration of experiment	Size of drops		
	Experiment I	Experiment II	Experiment III
90 mins.	636	636	945
150 ,,	644	643	947
210 ,,	649	648	969
270 ,,	652	651	974
330 ,,	654	653	978
390 ,,	655	654	980
470 ,,	655	654	980

It will be seen that by chance the drops in experiments I and II had the same length. The increases in length are in close agreement, and the corresponding results obtained by Bertrand's method are shown below :—

TABLE II.

Action of taka-diastase on starch at 24°.

B.—Bertrand's Method.

Duration of experiment mins.	60	120	180	240	300	360	420	480
Maltose, in c.c. of $KMnO_4$	10·10	12·50	14·20	15·80	16·30	16·50	16·65	16·70

The readings in column II are the mean values from duplicate experiments. The graphs (*Fig. I*) obtained on plotting the above results (Expt. I from A, and B) show a striking similarity and have been repeatedly confirmed.

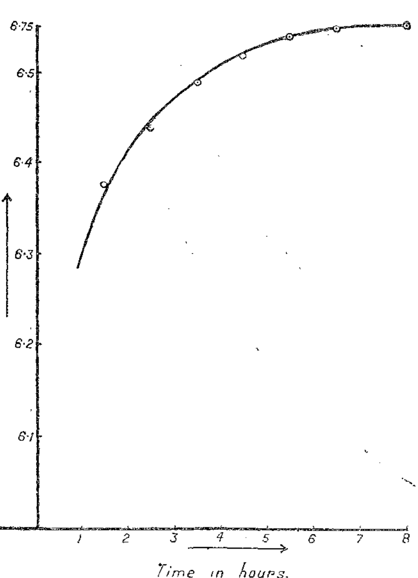
The activity of a sample of invertase prepared from autolysed yeast-liquor by Hudson's method has been followed by this micro-method. The experiment was conducted exactly as previously described, and the results observed are in fair accordance with those obtained by the polarimetric method.

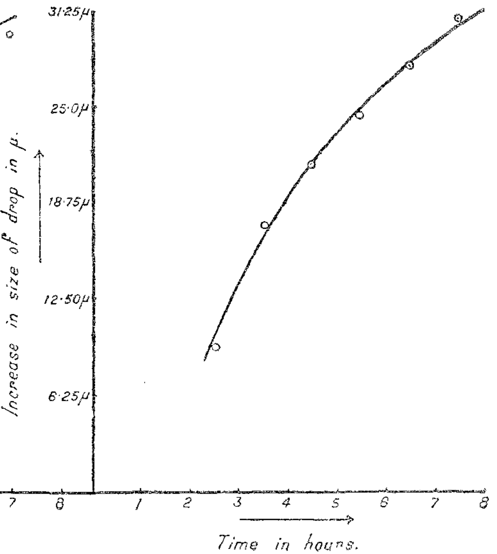
TABLE III.

Action of invertase on cane-sugar at 24°.

A.— Micro-Method.

Duration of experiment	Size of drops			
	Expt. I	Expt. II	Expt. III	Expt. IV
85 mins.	342	355	543	831
145 ,,	347	362	558	849
205 ,,	351	368	571	854
265 ,,	354	376	577	864
325 ,,	361	382	582	872
385 ,,	362	384	587	878
445 ,,	372	398	592	886
1320 ,,	462	472	672	965


Hydrolysis of Starch by Taka-diastase.
Bertrand's Method
Micro-method
Maltose Estimation C.C of KMnO4 used for 10cc. of Liquid
Size of the drop in drum revolutions
Time in hours.
Time in hours.



Hydrolysis of Sucrose by Invertase.
Polarimetric method
Micro-method
Polarimetric Reading in degrees
Increase in size of drop in μ.
31·25μ
25·0μ
18·75μ
12·50μ
6·25μ
Time in hours.
Time in hours.

TABLE IV.

Action of invertase on cane-sugar at 24°.

B.—Polarimetric Method.

Duration of experiment mins.	120	180	215	275	335	420
Rotation	—2·81°	—3·89°	—4·72°	—5·85°	—6·72°	—7·16°

The angles are the mean values of four polarimetric readings; these, and increase in the size of the drops, have been plotted separately against time. The graphs (Fig. II; Expt. III from *A*, and *B*) show a striking similarity and have been frequently reproduced.

CONSIDERATION OF PROBABLE ERRORS.

That the method is valid and applicable to enzyme work has been shown in a number of ways. In a closed chamber, such as is used in bacteriology for hanging drop cultures, there was placed a small quantity of the control starch solution, and the chamber was closed by a greased cover slip with a small drop of the active solution hanging on the under side. The drop was measured at regular intervals under low power.

TABLE V.

Duration of experiment mins.	60	90	112	165	230	250
Size of drop, in hundredths of a scale division ...	1364	1367	1385	1398	1404	1407

The results show that there is a gradual and steady increase in the size of the drop, as was expected; this increase diminishes with time, corresponding to a fall in the intensity of action during hydrolysis. A complementary experiment with the active solution in the chamber and a drop of inactivated solution on the greased cover slip showed a corresponding fall in the size of the drop.

In a capillary tube a drop of the control solution at first decreases in size and then becomes constant, a short time being required for the air-space of the tube to become saturated and equilibrium established. The figures given below indicate the extent of this change. In this case the equilibrium was attained in about half an hour.

TABLE VI.

Single drop of control solution.

Duration of experiment mins. ...	10	18	28	42	53	115
Size of drop	433	420	414	416	414	414

A single drop of the active solution, on the other hand, behaves rather differently. If the concentration of enzyme is low there will be at first a slight decrease pending saturation of the air on either side of the drop; then an equilibrium is reached followed by an increase in

the size of drop owing to deposition of condensed vapour from the saturated air spaces as the vapour-pressure of the active solution falls. Finally the size becomes constant. The duration of these stages depends on the degree of activity; the first stage is hardly noticeable in many cases, the increases and final equilibrium only being observed.

TABLE VII.

Single drop of active solution.

Duration of experiment mins. ...	5	25	30	35	45	125	235	355
Size of drop	495	493	492	492	496	497	500	500

The results show conclusively that the changes in the size of drop proceed in a regular and measureable way; also that the method based on these principles is valid and is applicable to enzyme hydrolysis.

Mixing of Liquids:— Using a solution coloured by a small quantity of potassium permanganate as standard it was invariably found that the neighbouring drop becomes perceptibly coloured sooner or later, the time depending on the diameter of the tube and the distance between the neighbouring drops. Hence a slight mixing does occur during the experiment. Barger has drawn attention to this point and has shown that the amount of mixing is approximately:

$$\frac{2\delta l}{rd}$$

where δ is the average thickness of the film adhering to the walls of the tube, which depends upon the surface-tension and viscosity of the liquid, and is presumably a constant for a given liquid; l is the distance travelled by the drop along the tube, i.e., the distance between the end of the tube and the final position of the drop; d, the average length of the drop and $2r$ the diameter of the tube. In order to minimise mixing, l should be made small and the drops should be allowed to traverse as short a distance as possible. The thickness of the drop must be as great as possible and so also the diameter of the tube but small enough to allow of the formation of stable drops by surface tension.

We have attempted to prevent mixing by various measures and of these the most effective seems to be the use of two drops only, one active and the other control, drawn in from opposite ends and leaving between them a small distance of the tube partly dry. In this way several experiments have already been conducted and the results seem to be very promising; but the disproportionate distance between the drops, about 2 cms., is a serious drawback. Another method, in some ways superior, is to introduce the liquid into the experimental capillary tubes by means of a very fine capillary. This method has certain

decided advantages over the last, among which a consideration of the distances between the drops is probably the most important.

Dilution:— The distillation of the solvent from the control to the active drop alters the concentration of the solution, first at the surface and gradually in the body of the liquid by diffusion. Yamakami has recognised this factor which, however, has not a great significance in his or Barger's work. It is not possible to avoid it, however. The change in the length of the drops after an interval of about eight hours was less than 10 per cent. in many cases and, assuming that enzyme activity is proportional to concentration of the solution, variation in activity will not be great. Moreover, the change is only gradual. It is for this reason that the readings in the last stages of the hydrolysis are not reliable, for then the question of dilution becomes relatively important.

Setting the tubes and fixing to the slide.—When all the drops are drawn into the tube they are allowed to move down until the last drop is about 1 c.m. from the end. The ends are then very carefully sealed, after which the tubes are transferred to a water-bath. For convenience in operating the microscope, it is necessary to fix the tubes to a slide. It has been found during the progress of the work that the use of paraffin wax, Canada balsam or goldsize is not without effect in bringing about temperature changes which influence the vapour-pressure. The result of using melted paraffin is to increase the temperature at the ends, and goldsize or Canada balsam lowers the temperature at the ends owing to the evaporation of the solvent. The error may be small but is always present. It was thus imperative to avoid such fixing agents, and a rubber-band has therefore been used to keep the tubes in position on the slides.

Bacterial Contamination:—Experiment showed that bacterial contamination occurs. To ascertain the magnitude of the effect, five small conical flasks (50 c.c.) were provided with 20 c.c. of starch solution containing a small quantity of enzyme, and immediately sterilised. Four of the flasks when cool were deliberately infected with *aspergillus oryzæ*; all five were then transferred to a desiccator containing alkaline pyrogallol and thus maintained under unaerobic conditions. The flask not infected served as a control. From time to time the flasks were removed and 10 c.c. used for the estimation of reducing sugars by Bertrand's method, using $N/25$ permanganate for titrations. The results are as follows:—

TABLE VIII.

Duration of experiment ...	Control ...	9 hrs.	32 hrs.	51 hrs.	77 hrs.
$KMnO_4$ c.c.	15·67	15·84	16·10	20·02	21·60

The experiment shows that the saccharifying effect in the first eight hours, the duration of our experiments, is practically negligible.

Distance between the drops.—It has been pointed out, that the distance travelled by the drops along the tube should be as small as possible in order to minimise mixing while [illegible] the tubes. This means that the distance between two neighbouring drops should be as small as possible, but not so small that they coalesce and mix. Moreover, proximity of the drops appears to favour rapid interchange of vapour; and the success of the experiment requires instantaneous attainment of equilibrium, coincident with the hydrolysis. Further, if the distance is very great a rapid exchange of vapour with small differences in concentration does not in practice take place, for no apparent theoretical reason.

LIMITATIONS.

The method is not universally applicable. When gases and volatile liquids accompany enzymic processes the micro-method must obviously fail. Zymase, which produces carbon dioxide and alcohol from glucose, also the oxidases and peroxidases, for instance, cannot be studied by this method.

We do not claim that a high degree of accuracy is obtainable by this method, but it does give results of value and provides a useful and yet simple quantitative way of following enzymic activity even when only minute quantities of enzymes are procurable. The possibilities of an extended application of the method are easily conceivable. It is not only possible to detect the common enzymes in such complexes as cell-saps, but it is also possible to estimate their activities individually. The former is achieved by providing various substrates and observing the regular changes in the size of the drops, noticeable only in those undergoing chemical action.

On the quantitative side of the work, since the fluid itself may contain substances liable to change, there is conducted a blank experiment in which the boiled fluid is alternated with untreated fluid and suitable corrections applied.

SUMMARY.

1. A quantitative microscopic method is described for determining the activity of the more common enzymes.

2. A comparison of the results obtainable by this method with recognised standard methods has been made.

3. Factors which influence the accuracy of the method have been discussed at length and measures for improvement suggested.

4. It is pointed out that the particular value of the method lies in the fact that the enzymes can be detected, characterised and estimated even when only minute quantities are available.

In conclusion our grateful thanks are due to Professor R. V. Norris for his kind encouragement and keen interest throughout the progress of the work. Our thanks are also due to Mr. B. N. Banerji for many helpful criticisms.

Department of Bio-Chemistry,
Indian Institute of Science,
Bangalore.

[Accepted, 16-11-27.]