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A MANOMETRIC ADAPTATION FOR THE RAPID
MICROBIOLOGICAL ASSAY OF VITAMINS AND
AMINO ACIDS*

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Recently, Siu and Mandels (1, 2) published a manometric method for a rapid assay for growth wherein growth of the organism is measured by determining the respiratory oxygen consumption. They demonstrated the applicability of the technique to the evaluation of the susceptibility of different materials to microbiological degradations and to the assay of fungitoxic substances. They suggested the feasibility of adapting this method to the biological assay for vitamins, amino acids, etc., and this is the object of the present paper. In 1944, Atkins, Williams, and Frey (3) reported on a manometric procedure employing the Warburg apparatus for vitamin estimation with lactic acid bacteria. Unfortunately, Warburg manometers are not well adapted to routine assay, whereas the manometers used herein can be easily manipulated by untrained technicians. Essentially, they are a macro-modification of the Barcroft-

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Haldane differential manometers; 250 ml flasks are used as vessels and are connected to the manometers by means of flexible tubing. Since a closed system is used, the measurements are not affected by fluctuations of atmospheric pressure, and precise temperature control is not mandatory.

Lactic acid bacteria are the organisms most commonly used for the microbiological assay of vitamins and amino acids. In their growth on glucose the metabolism of these organisms is predominantly fermentative as opposed to respiratory, and lactic acid production is much greater than oxygen absorption. Hence, manometric estimation of growth is accomplished more advantageously by determining the carbon dioxide liberated from a bicarbonate buffer by the lactic acid formed than by measuring O₂ consumed (4). Certain growth substances such as choline, inositol, and *p*-aminobenzoic acid can best be determined by the growth response of certain mutant forms of fungi (5, 6). The fungi are then harvested, dried, and weighed. This procedure is both time consuming and tedious. It can be avoided by the use of manometers, and, in this case, oxygen consumption is preferentially measured. With this procedure the time for the determination is shortened from 72 hours to 18 hours.

METHODS

The manometers.—Details of the construction of the manometers have been previously given (1). To measure O₂ consumption, the carbon dioxide evolved in respiration is absorbed by potassium hydroxide in a well. This procedure is applicable to methods where fungi are used. In the determinations using bacteria where CO₂ evolution is measured, the cup containing the alkali is omitted.

Assay procedure for valine.—The determination of valine is chosen for description as one of the several successful applications. The experience with assays for methionine and aspartic acid parallel it in general. *Streptococcus faecalis* 9790 was used with the medium reported by Baumgarten *et al.* (7). The standard curve obtained from measurement of turbidity (as produced in test tubes when incubated 30 hours at 37°C.) was run simultaneously with that of the curve for the response as measured by manometers mounted in incubators maintained at 30°C. The medium in the 250 ml flasks attached to the manometers differed from that in the test tubes (5 ml basal medium plus 5 ml water) in that 5 ml of 0.2 *M* sodium bicarbonate solution was used in place of the water. This amount of bicarbonate was calculated to be twice the maximum amount, based on the titrimetric values, required in a valine assay. The pH of the basal medium in the test tubes was 6.8; that in the flasks after addition of the bicarbonate was 8.4. The inoculum used in the standard test tube set-up was the normal amount, viz., one drop per tube, wherein the 24 hour growth in 10 ml of complete medium was centrifuged and washed twice

with 5 ml of sterile saline, and finally made up to approximately 2 ml. In the case of the manometer flasks, the 24 hour cultures of the organism were washed twice and suspended in 30 ml of saline, and 1 ml of this suspension was added to each of the flasks. This represents approximately 130 times the normal amount added to the test tubes and was purposely done to step up the rate of acid production. (Initial turbidity of the solution is unimportant when using the manometric technique.)

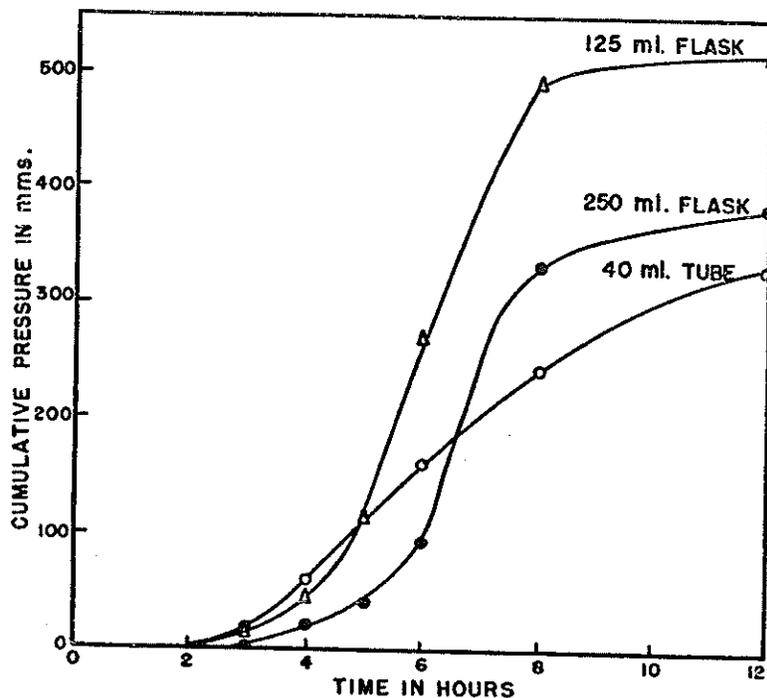


FIG. 1.—Course of lactic acid production by *S. faecalis* grown in 3 different size vessels.

Assay procedure for choline.—The standard curve was obtained by the method of Horowitz and Beadle (5), using the *choliness* mutant of *Neurospora crassa*. After 72 hours growth at 30°C., the stationary cultures were filtered off on tared filter papers and weighed after drying in a vacuum oven for 2 hours at 50°C. In the manometric method, a small cup containing 1.5 ml of 10% potassium hydroxide and a filter paper wick was suspended in the flasks. This absorbed the evolved carbon dioxide and the manometers recorded O₂ pressure changes. The inoculum was prepared by adding sterile water to a culture of the fungus until the resultant turbidity gave a reading of 200 on the Klett-Summerson photocolormeter when a red filter having a maximum at 640 m μ was used.

For the standard test, 1 drop of the suspension was added, and in the manometric test, 1 ml was added.

The increase in time consumed in the preparation of the equipment for the manometric procedure as compared to the test tube method is negligible. The additional time is simply that of attaching the inoculated flasks to the manometers by means of their ground-glass joints. Aseptic techniques are used throughout in both procedures.

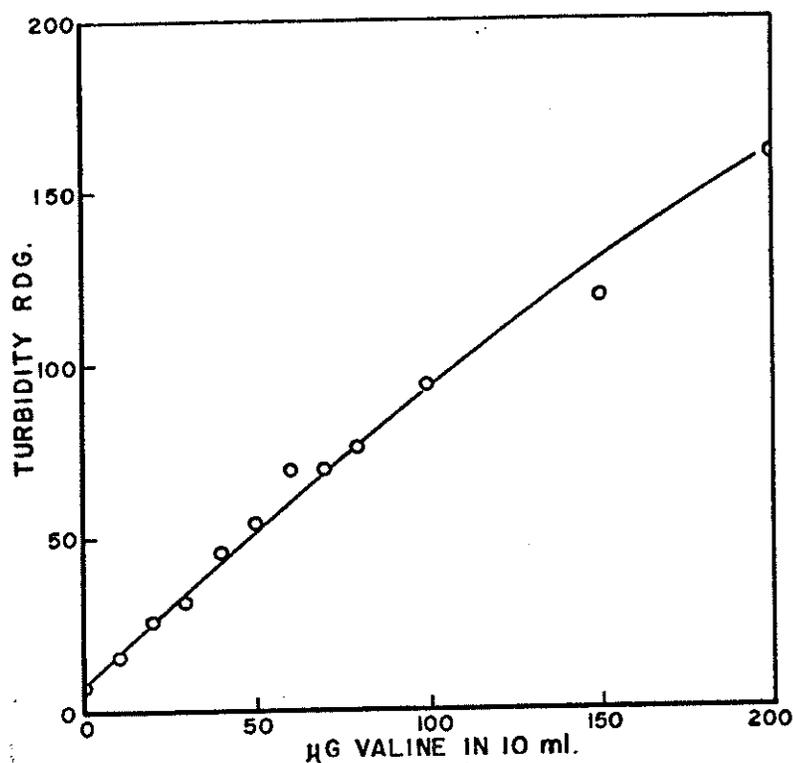


FIG. 2.—Typical standard curve obtained for d-valine. Time = 30 hours.

RESULTS

Choice of vessel size to be used with manometers.—Originally, Siu and Mandels (1, 2) used a 250 ml Erlenmeyer flask in their study of mildew susceptibility of fabrics, etc., mainly because it allowed exposure of a relatively large fabric surface. The manometer was then designed to accommodate the expected pressure differences. Since the manometers are fixed in size, it was of interest to determine whether different sized vessels were necessary for the 10 ml total volume of basal medium for bacterial growth and the 20 ml used for fungal growth. Figure 1 shows the growth

curve obtained with *Streptococcus faecalis* at 37°C. using 40 ml tubes and 125 and 250 ml Erlenmeyer flasks. The 250 ml flask was used thereafter, since (A) it allowed the pressure curve (growth period) to level off before that of the 40 ml tube; (B) the pressure changes were not so great as those in the 125 ml flask and required less resetting of the manometers. As designed, the manometers can register a pressure of approximately 200 mm before it becomes necessary to equalize the pressure in both flasks to avoid forcing over the manometer fluid. Use of a manometer fluid of

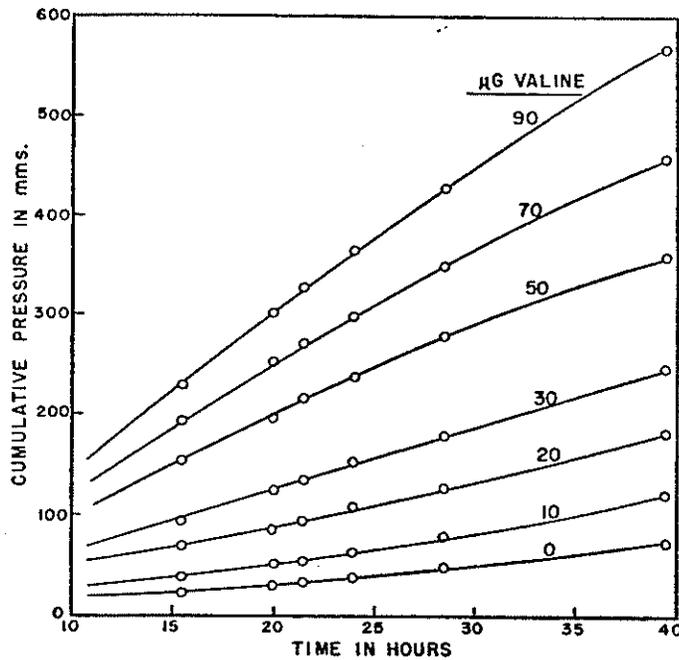


FIG. 3.—Lactic acid production by *S. faecalis* in response to varying amounts of valine.

greater density than the Brodies solution used herein would avoid this difficulty (8).

At the same time that a standard curve for the growth response of *S. faecalis* to varying amounts of valine was determined by turbidimetric measurements, a series of time-pressure curves, with varying amounts of valine, were prepared. These are illustrated in Figures 2 and 3. In all cases they are averages of duplicate readings. Figure 4 is a translation of the values of Figure 3, and show cumulative pressure against micrograms of valine after a certain time of incubation. Simultaneously, a lactalbumin hydrolysate containing 11.2% nitrogen was run at suitable dilutions with both the test tube and manometric techniques. In the latter case,

however, due to lack of sufficient manometers, only 3 dilutions could be run. Using the resultant turbidimetric curve, calculations to a moisture-free basis gave 5.98 per cent of l-valine. This agrees well with a series of other values found in the literature (9) for lactalbumin. An average of 223 micrograms of dl-valine per ml of test lactalbumin was obtained from the turbidimetrically measured curve. The average for the 3 manometric

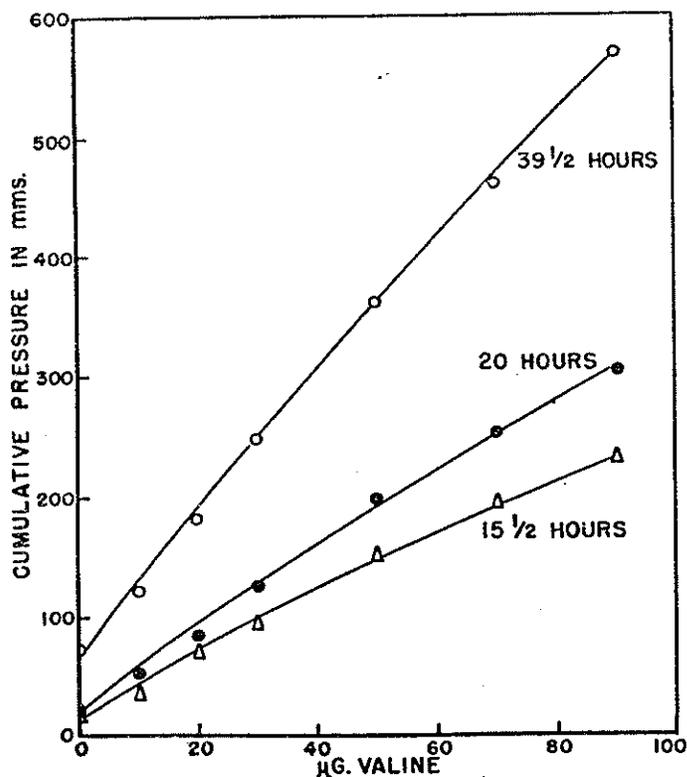


FIG. 4.—Effect of valine on lactic acid production.

values, taken from the curves of Figure 4, are: 15½ hrs., 237.5 micrograms; 20 hrs., 240 micrograms; and 39½ hrs., 204.4 micrograms.

In another series of experiments with valine, a set of curves similar to that in Figure 2 was obtained but with points at 4, 5, 6, 8, 12, and 22 hours. A 10 hour and 11½ hour plot of the cumulative pressure against micrograms of valine as shown in Figure 5 gave smooth curves similar to those in Figure 4.

The effect of choline concentration on growth of the fungus *Neurospora crassa* is given in Figure 6, and was determined by the dry weight of my-

celium at 3 days. The course of respiration as determined on the manometers is given in Figure 7. One hundred and twenty-five ml flasks were used in this experiment. An earlier change in the course of respiration might have been achieved if the spore suspension used as inoculum had been aseptically washed several times with distilled water. This would have prevented possible carry-over when this relatively large volume of inoculum was used. In Figure 8, the data of Figure 7 are replotted, with time constant and the amount of choline varied.

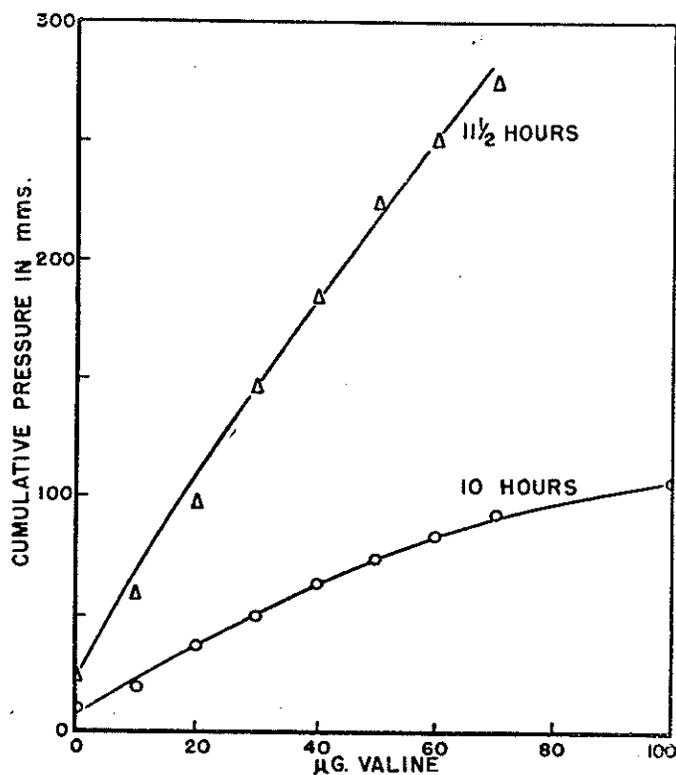


FIG. 5.—Lactic acid production by *S. faecalis* with varying quantities of valine.

DISCUSSION

The primary objective of this study was to determine the feasibility of applying the manometric technique of Siu and Mandels (1, 2) to the microbiological assay of amino acids and vitamins. Limitations of both time and number of available manometers precluded extensive application. However, sufficient data have been accumulated to show that manometers can be used for this purpose. Refinements such as vessel size, size of manometer capillary, specific gravity of manometer fluid, size of inocu-

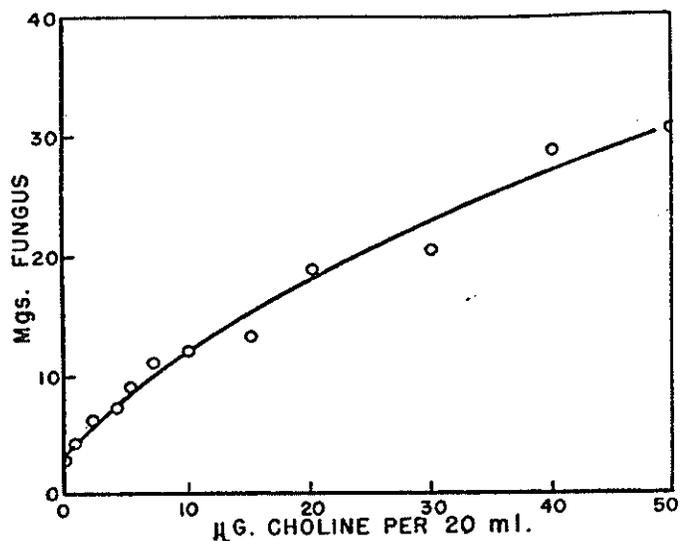


Fig. 6.—Dry weight of cholineless fungus after 3 days as a function of concentration of choline in the medium.

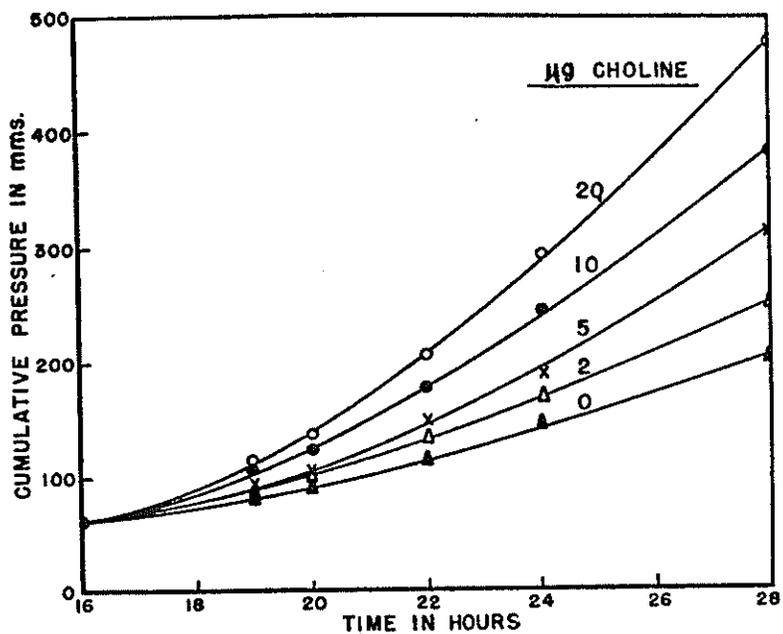


Fig. 7.—Course of respiration of *Neurospora crassa* in response to varying amounts of choline.

lum, volume of basal medium, buffers, temperature and other variable factors would improve the precision.

Preliminary experiments indicate that it is possible to reduce the number of manometers necessary to run an assay on an unknown substance by setting up one series of perhaps 5 manometers and placing a constant amount of the standard on the right side vessel. Then the pressure gener-

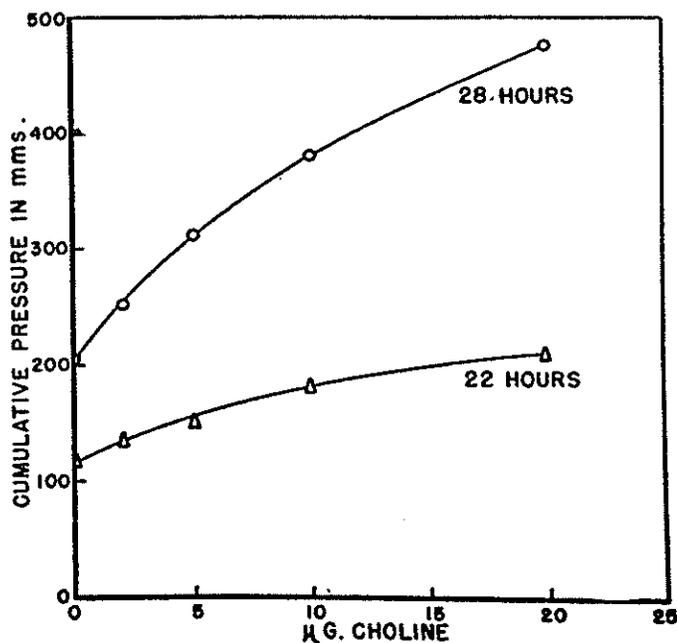


FIG. 8.—Effect of choline on the oxygen consumption.

ated by the presence of this quantity opposes the pressure generated by the effect of the 5 dilutions of the unknown on the left. These latter dilutions should be so made up as to bracket the quantity of standard used. Values should be taken only from the straight line portion of the normal growth curve.

Since respiration or fermentation of the microorganisms used correlates with their growth as measured by turbidity, titration, or direct weighing (3, 4), advantages of the manometric method may be pointed out. When using bacterial growth methods, the manometric adaptation should allow determination of amino acids or vitamins in crude materials, particularly in the case wherein turbidity cannot be avoided in the preparation of the samples for assay. One example would be in the vitamin B₁₂ assay of chicken feeds; others in the various vitamin assays of distillery mashes and Army concentrated rations.

A reduction of the time factor for assay is in some cases extremely desirable and can be obtained by the use of manometers. For example, Figure 4 shows that in 15½ hours (approximately half the time needed to produce a good turbidity), a standard curve was derived which allowed a fairly satisfactory determination of valine in lactalbumin. It is possible to cut this down even further with the above suggested refinements. The weighing of mycelium in those methods which use a fungus as the micro-organism is a rather tedious procedure. Here too, time may be cut from 3 days to 1 day, as indicated by the curves in Figure 8.

SUMMARY

1. A manometric method has been devised which is applicable to the determination of amino acids, vitamins, and accessory growth factors.
2. The method is based upon a determination of growth as measured by carbon dioxide pressure generated as the result of release of lactic acid by the bacteria; in the case of assays using fungi, it is based upon determination of growth as measured by oxygen consumption.
3. The manometric procedure eliminates the necessity of filtering off, drying, and weighing the fungi which are used for certain assays. Elapsed time in this case may be cut from 3 days to 1 day.
4. The time in the case of bacterial growth responses may be lowered from 30 hours to at least as low as 11 hours.

ACKNOWLEDGMENTS

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