RESPIRATORY ENZYME SYSTEMS IN SYMBIOTIC NITROGEN FIXATION

I. The "Resting Cell" Technique as a Method for Study of Bacterial Metabolism

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Studies on the biochemistry of symbiotic nitrogen fixation (Wilson and Fred, 1937) indicate that oxidation reactions play a significant, though probably indirect, rôle in this process. A survey of the properties of the respiratory enzymes in species of the root-nodule bacteria (*Rhizobium sp.*), both when grown alone and with the proper host plant, appears necessary for progress on the problem of the mechanism of the reaction. Previous studies of the respiratory activities of the root-nodule bacteria (Georgi and Wilson, 1933; Neal and Walker, 1935, 1936; Thorne and Walker, 1935; Thorne, Neal and Walker, 1936; Walker, Anderson and Brown, 1934, a, b) were made primarily with growing cultures. Interpretation of the data is difficult since effects on growth are not readily differentiated from effects on respiration.

The "resting cell" techniques eliminate many of the objections associated with studies made on growing cultures (Konishi and Tsuge, 1934). Resting cells, or more accurately, non-proliferating cells are washed suspensions of bacteria which are used as a source of enzymes for studies on bacterial metabolism. The enzyme is not separated from the cell but is "isolated" through use of specific substrates, inhibitors and other means. There is evidence (Quastel, 1928, 1932; Stephenson, 1930) that certain

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enzymes concerned with energy-liberating reactions consist primarily of definite patterns of molecules in the cell or on its surface. The activity of such enzymes is a function of the orientation of these molecules in the surface. Separation of this type of enzyme from the cell by the usual methods would be a self-defeating procedure, as, obviously, dissolution of the cell would result in destruction of the enzyme. Resting cells are of particular value for the study of reactions catalyzed by such "structure bound," enzymes.

Some other advantages of the use of resting cells may be cited:

1. Respiratory activities are separable to a considerable extent from those associated with growth. Since no source of nitrogen is supplied, proliferation of the cells is at a minimum, and the reactions are greatly simplified. With growing cultures, separation of growth and respiration responses is difficult and frequently impossible.

2. The kinetics of a reaction can be readily followed. Consideration of the *rates of reaction* rather than *total reaction* is frequently advantageous and usually desirable for accurate interpretation of results (Werkman, 1927; Burk, 1934).

3. Single reactions may be studied rather than the combined effects of several. If formate is the sole substrate present, then resulting changes arise from decomposition of formate. This "isolation" of specific reactions is of great value for the investigation of possible intermediates in the decomposition of a given substrate. Such isolation is usually difficult with media which allow growth.

4. Closely related reactions often may be differentiated by use of specific inhibitors or by determination of the characteristic properties of the enzyme systems concerned with the reactions.

5. Results are referred to a standard which allows the data from one experiment to be directly compared with those from another. For this purpose the constant, Q_{O_2}, defined as the cubic millimeters of oxygen consumed per hour per milligram dry weight of organism is usually employed. As this constant represents a *rate per unit weight*, the original data are corrected for differences in the mass of organisms in various suspensions.

Similar constants can be calculated for uptake or disappearance of substances other than oxygen. If growing cultures are used, it is difficult to correct for differences in number or mass of organisms since the values change with time.

6. The short-time nature of the experiments eliminates effects arising from differences in development of cultures. Also, once the technique has been developed, many experiments can be made in a single day, as the actual measurements usually require an hour or less.

The cited advantages are exemplified by the outstanding investigations on bacterial metabolism of the Cambridge University group. Solution of many of the problems attacked by this group of workers would have been delayed if the conventional methods of bacteriology had been employed. Among these are: the resolution of the hydrogen enzymes of *Escherichia coli* (Stephenson and Stickland, 1931; Stephenson, 1937; Woods, 1936); the manner in which some strict anaerobes secure their energy (Stickland, 1934); adaptive *versus* constitutive enzymes (Stephenson and Yudkin, 1936; Stephenson and Gale, 1937; Quastel, 1937).

Although for the past decade English investigators have employed the resting cell techniques for study of bacterial metabolism, similar investigations in America are rather uncommon. Werkman and his collaborators (Stone and Werkman, 1937; Wood, Stone and Werkman, 1937) used non-proliferating cells for studies of intermediates in bacterial fermentations. Others employed such suspensions for investigations of the dehydrogenases of bacteria using the methylene-blue technique (Kendall and Ishikawa, 1929). However, detailed unvestigations of specific enzyme systems, through use of the micro-respirometer together with the methylene-blue technique and isolation of chemical compounds have been quite neglected. Part of the delay, no doubt, arises from the slow adoption of the micro-respirometer for metabolic studies. The future will probably witness increasing replacement of the traditional methods for study of bacterial metabolism by techniques using resting cells.

This paper suggests the type of problem which may be advantageously undertaken through use of the resting cell techniques

and details the preliminary survey that must be made when the metabolism of a new group of organisms is investigated. The actual data will be of chief interest to those studying the specific organism used, but the same general procedures are applicable to a study of almost any group of microörganisms.

METHODS

The measurement of oxygen uptake with the Warburg or the Barcroft respirometer has been described (Dixon, 1934). The papers of the Cambridge workers already cited should be consulted for details of the general procedures for preparation of the suspensions of bacteria and for determination of methylene-blue reduction. *Dry weight* was estimated by drying 2 cc. of the suspension at 100°C. and deducting the weight of the salts in the suspending solution. *Total nitrogen* was determined by the modified Pregl micro-method or by the semi-micro method of Umbreit and Bond (1936). *Hydrogen-ion* estimations were made with the glass electrode. Wisconsin strain 209 of *Rhizobium trifolii* was used as the test organism. Purified chemicals were used in the media and special chemicals (Kahlbaum or Eastman) as the substrates. Both the Barcroft and the Warburg types of micro-respirometer were employed.

PREPARATION OF SUSPENSIONS

Before actual measurements of the respiratory activities of an organism are undertaken, methods for preparing a suspension with satisfactory properties must be developed. These include the following points: (*a*) the organisms should be readily separable fron the suspending or washing solutions; (*b*) the cells should possess a high respiration rate, otherwise the quantity necessary for estimation of oxygen uptake may introduce complicating factors; (*c*) the *endogenous* metabolism, that is, the respiration of the organisms alone, should not be excessive; (*d*) the rate of respiration should be directly proportional to the number of viable organisms present; (*e*) the activity of the suspension should be stable, otherwise it is necessary to prepare a fresh suspension for each determination.

The first attempts to prepare a satisfactory suspension of *R. trifolii* failed. The organisms were grown on medium 79, a yeast-extract mineral-salts mannitol agar substrate (Fred, Baldwin and McCoy, 1932), washed from the agar surface with Allison's solution[2] (Allison and Hoover, 1934), and a suspension prepared by the usual technique. The rate of respiration on glucose of this suspension was very low, the Q_{O_2} being less than 10. Most bacteria have a Q_{O_2} on glucose of 20 to 50, and a few species may exceed 100. Recovery of the organisms from the suspending solution was very poor even with prolonged centrifuging. Moreover, the endogenous respiration of the cells was about 60 per cent of that on glucose. Such preparations are almost useless for study of oxygen uptake on different sources of carbon since most of the respiration arises from cellular constituents and not added substrate.

The cause of the difficulties was the production by the bacteria of a gum which is not readily removed from the cell. This gum gave the organisms colloidal properties which interfered with their separation from liquids, it lowered the Q_{O_2} of the cells since their dry weight included adhering gum, and it provided a substrate for the organisms which resulted in a high "endogenous" respiration. The cells were very low in percentage of nitrogen; if the rates of respiration were calculated on basis of nitrogen content, the values were comparable with those for other microorganisms.

Apparently the difficulties would be overcome if the nitrogen content of the cells could be raised. This was accomplished by growing them on a yeast-extract mineral-salts agar without added carbohydrate. On this medium the organisms grew readily, produced little gum, were easily separated from suspending and washing media, were high in percentage nitrogen and possessed satisfactory respiratory activity.

Both total crop of organisms and percentage of nitrogen in the cells increased as the quantity of yeast extract in the medium was raised from 0.25 to 1.0 per cent, but further increases had

[2] Preliminary experiments indicated that this solution of balanced salts gave better suspensions of *R. trifolii* than did the customary normal saline solution.

little effect. In subsequent experiments the medium consisted of one-per-cent yeast extract, the salts of medium 79 and 2.5 per cent agar. The agar was increased from 1.5 to 2.5 per cent as this gave the medium physical properties which aided in the removal of the cells from the surface.

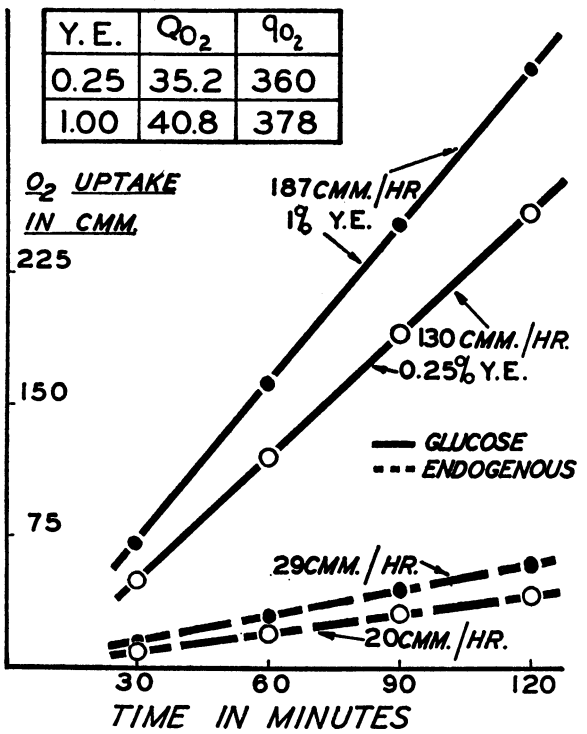

FIG. 1. EFFECT OF CONCENTRATION OF YEAST EXTRACT IN MEDIUM ON RESPIRATION OF R. TRIFOLII

Dry weight of suspension grown on 0.25 per cent yeast extract was 3.7 mgm. per cubic centimeter; nitrogen content, 0.362 mgm. per cubic centimeter. Dry weight of suspension grown on 1.0 per cent yeast extract, 4.6 mgm. per cubic centimeter; nitrogen content, 0.495 mgm. per cubic centimeter.

Respiration data from two suspensions are presented in figure 1 which illustrates the method for calculating the Q_{O_2}. The oxygen uptake of a bacterial suspension is plotted against time, and the slope of the resulting line is determined. Theoretically, it requires only two points to determine this slope, but in practice

at least four points are taken in order to make sure that the rate of respiration is constant. The slope of this line in cubic millimeters per hour divided by the *dry weight* in milligrams of the organisms in 1 cc. of suspension gives the Q_{O_2}.

Values of the Q_{O_2} increased with concentration of yeast extract in the medium, probably because of a decreased gum formation. If the *nitrogen content* of the organism were used to calculate a constant, q_{O_2}, defined as the oxygen uptake in cubic millimeters per hour per milligram N in the cells, the values obtained were essentially independent of gum production. Because of the more stable character of the constant based on nitrogen content, this datum was used rather than the traditional Q_{O_2}. As can be seen in the figure, the endogenous respiration of the organisms grown in the absence of added carbohydrate constitutes only 15 to 20 per cent of the respiration on glucose.

During endogenous respiration, NH_3 was liberated, but none was formed by cells given a source of carbon. This result suggests that endogenous respiration represents, at least in part, oxidative deamination of cellular amino acids and that this type of oxidation does not occur with cells given a source of readily available carbon (protein-sparing action of carbohydrates). For this reason the oxygen uptake of cells in the absence of substrate was not subtracted from that in the presence of substrate before calculation of the q_{O_2}.

As shown in table 1, the addition of sucrose to the basic yeast-extract medium caused the percentage of nitrogen in the cells and the Q_{O_2} to decrease, and the endogenous respiration relative to that with glucose to increase. Also, as addition of sucrose caused a greater production of gum, the efficiency of separating the cells from the suspending liquid was decreased. To the eye, the largest crop was formed in the presence of the higher concentrations of sucrose, but the greatest crop recovered on centrifuging and washing was obtained from the medium with the lowest level of carbohydrate.

Since all the effects of adding sucrose are undesirable insofar as the respiratory activity of the resulting suspensions are concerned, no carbohydrate was used in the standard medium adopted. In

general, growth of the organism in the absence of added carbohydrate should be advantageous since respiration studies on different sources of carbon would not be complicated through previous "adaptation" of the bacteria to a specific carbohydrate. If a preliminary adaptation is wanted, as much as 0.1 per cent carbohydrate can be added to the medium used to grow *R. trifolii* with little loss in the desirable properties of the suspension.

The data in table 2 show that the optimum time for harvest is about 48 hours since at this time the q_{O_2} has decreased only

TABLE 1

Effect of addition of sucrose to yeast extract medium on properties of suspensions of R. trifolii

SUCROSE ADDED	CROP YIELD PER CUBIC CENTIMETER			RATE OF RESPIRATION	Q_{O_2}	q_{O_2}	ENDOGENOUS RESPIRATION
	Dry weight	Total N	Per cent N				
per cent	*mgm.*	*mgm.*		*cmm. per hr.*			*per cent**
0.05	3.50 3.45 4.00	0.348 0.348 0.318	9.26	139 138	37.9	410	15.3
0.10	4.00 3.30 3.25	0.266 0.259 0.259	7.42	116 113	32.5	438	16.6
0.25	3.40 3.20 3.60	0.208 0.259 0.222	6.76	100 96	28.8	426	24.0

* Per cent of total respiration on glucose.

slightly from the maximum obtained at 24 hours, and the total crop of cells has almost reached the maximum. The percentage nitrogen in the cells increased, and the endogenous respiration relative to that on glucose decreased with age of the culture. Both observations suggest that part of any carbohydrate-like material present in the yeast extract is converted into gum early in the development of the organism, and with the exhaustion of this readily available source of carbohydrate, the gum is utilized (cf. Georgi and Wilson, 1933).

As a result of these preliminary investigations, the following

method was adopted for preparation of suspensions of the organisms:

Technique for preparation of suspension. Stock cultures of *R. trifolii* are kept on a medium consisting of the mineral salts of Medium 79 plus 0.5 per cent Difco yeast extract (9 per cent nitrogen) and 1.5 per cent agar. The growth of a 48-hour-old

TABLE 2

Effect of age of culture on properties of suspensions of R. trifolii

AGE OF CULTURE	YIELD OF CROP				RATE OF RESPIRATION	Q_{O_2}	q_{O_2}	ENDOGENOUS RESPIRATION
	D.W. per cc.	N per cc.	Per cent N	Total N* in crop				
hours	*mgm.*	*mgm.*		*mgm.*	*cmm. per hour*			*per cent*†
24		0.208 0.185 0.178		0.190	92 92 94		485	21.3
48	5.80 5.70 5.65	0.490 0.490 0.460	8.41	2.41	203 203 209	35.8	428	18.6
72	6.20 5.90	0.630 0.645 0.636	10.51	3.18	189 189 187	31.1	295	16.4
96	4.10 4.05	0.437 0.445 0.445	10.82	2.95	86 85 80	20.6	190	16.2

* Per bottle basis:
24 hours—organisms from 6 bottles made up to 6 cc.
48 hours—organisms from 4 bottles made up to 20 cc.
72 hours—organisms from 4 bottles made up to 20 cc.
96 hours—organisms from 3 bottles made up to 20 cc.
† Per cent of total on glucose.

slant is washed with 10 cc. of sterile Allison's solution into a sterile flask provided with sterile beads. Two cubic centimeters of a uniform suspension from several slants is spread over 100 cc. of a one-per-cent yeast-extract mineral-salts 2.5-per-cent-agar medium contained in a liter Roux bottle. A litmus milk tube is also inoculated with one drop of the suspension in order to test

the purity of the culture (Fred, Baldwin and McCoy, 1932). After incubation at 28° to 30°C. for 48 hours, the organisms are harvested. Ten to 15 cc. of sterile Allison's solution are added to each bottle, and the organisms transferred with the aid of a sterile glass rod into a sterile bottle containing beads. Organisms from replicate Roux bottles are combined and after thorough mixing are filtered through sterile glass wool to remove clumps. They are then centrifuged for 20 minutes at 3000 r.p.m., and after removal of the supernatant liquid are washed twice with sterile Allison's solution. The washed organisms are then suspended using about 10 cc. of sterile Allison's solution for each Roux bottle in the harvest. The suspension is kept at 3°C. in a 38 by 200 mm. pyrex test tube provided with means for aeration with aseptic precautions. Before use, the culture is aerated for 15 minutes at 0°C. One cubic centimeter of this suspension which contains 2 to 3 mgm. dry weight (about 0.25 mgm. N) is used for measurement of oxygen uptake or for reduction of methylene blue.

TESTS ON THE SUSPENSIONS

A desirable property of suspensions is that the organisms are in a state of maximum activity. Suspensions prepared by the technique just described met this requirement as the cells were in the log phase of development. If combined nitrogen and a source of energy were supplied to the organisms, the rate of respiration became a logarithmic function of time within a few hours (figs. 2 and 3). Allison and Hoover (1934) have described a factor which stimulates the growth of rhizobia in synthetic media. They claim that it acts as a coenzyme for respiration and have called it "Coenzyme R." Thorne and Walker (1936a, b) dispute that the factor is essential for growth or respiration and propose that the term used by Allison and Hoover be discarded in favor of "accessory growth substances." Since yeast extract is an excellent source of the accessory substance, the suspensions of *R. trifolii* used should be well supplied with the factor. That this view is correct was demonstrated in these growth experiments by adding to certain of the suspensions 100 p.p.m. of "Coenzyme R" prepared from *Azotobacter* (Hoover

and Allison, 1935). As can be seen in figures 2 and 3, such addition was without appreciable effect on the rate of growth, as

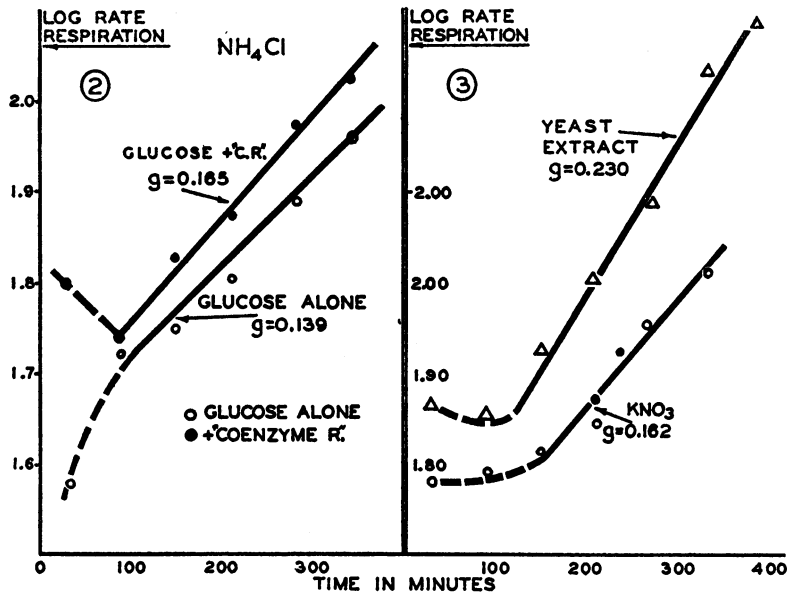

FIG. 2. EFFECT OF ADDITION OF NH_4Cl AND ACCESSORY GROWTH SUBSTANCES ("COENZYME R") ON RESPIRATION OF SUSPENSIONS OF R. TRIFOLII

FIG. 3. SAME AS FIGURE 2 WITH YEAST EXTRACT AND KNO_3 AS SOURCES OF NITROGEN

measured by increase in respiration. The values of the specific constant,

$$g = \frac{2.303}{t_2 - t_1} \log \frac{\text{respiration rate at } t_2}{\text{respiration rate at } t_1}$$

in which t refers to time, are probably identical within experimental error. That the respiration of these organisms is independent of added accessory substances ("Coenzyme R") was verified by adding 10 to 200 p.p.m. of the preparation to resting cells; no effect on the q_{O_2} could be detected.

Respiration of the organisms in these suspensions is directly proportional to the mass of cells. As shown in table 3, dilution of the suspensions as much as four-fold did not affect the q_{O_2}.

Suspensions may accordingly be directly compared without adjusting to some standard density of organisms. The data in the second part of table 3 show that the respiratory system of the cells is quite stable. The q_{O_2} on glucose of a suspension remains constant for at least 132 hours if kept in a refrigerator (3°C.). Such a property is very convenient, as a stock suspension can be used for tests made over a period of several days.

TABLE 3

Effect of dilution and storage of suspensions on rate of respiration (q_{O_2})

TREATMENT	SUSPENSION 2	SUSPENSION 3	SUSPENSION 4	SUSPENSION 5
Effect of dilution*				
cc.				
2.0	123			
1.0	132	250	354	
0.5		230		
0.25		250	353	
Effect of storage†				
hours				
0	127	215		411
12			354	
24	133	224		409
48				364
60		243	378	
84			353	
108			372	
132			368	

* Volume of suspension taken for measurement of q_{O_2}.

† Suspensions kept in ice-box at 3°C.

EFFECT OF ENVIRONMENTAL FACTORS

Once the chief factors concerned with the preparation of a satisfactory suspension have been established, the next step is to determine the proper technique for making the respiration measurements.

Effect of concentration of phosphate. In a typical experiment the following values for the q_{O_2} were obtained with mixed mono- and di-potassium phosphate buffers of different molality: M/3.33,

294; M/10, 387; M/30, 470; and M/90, 487. These data indicate that a definite decrease in the q_{O_2} occurs even with a buffer of phosphate molality as low as M/10. Because of this sensitivity of the organisms to high phosphate concentrations, and as lower

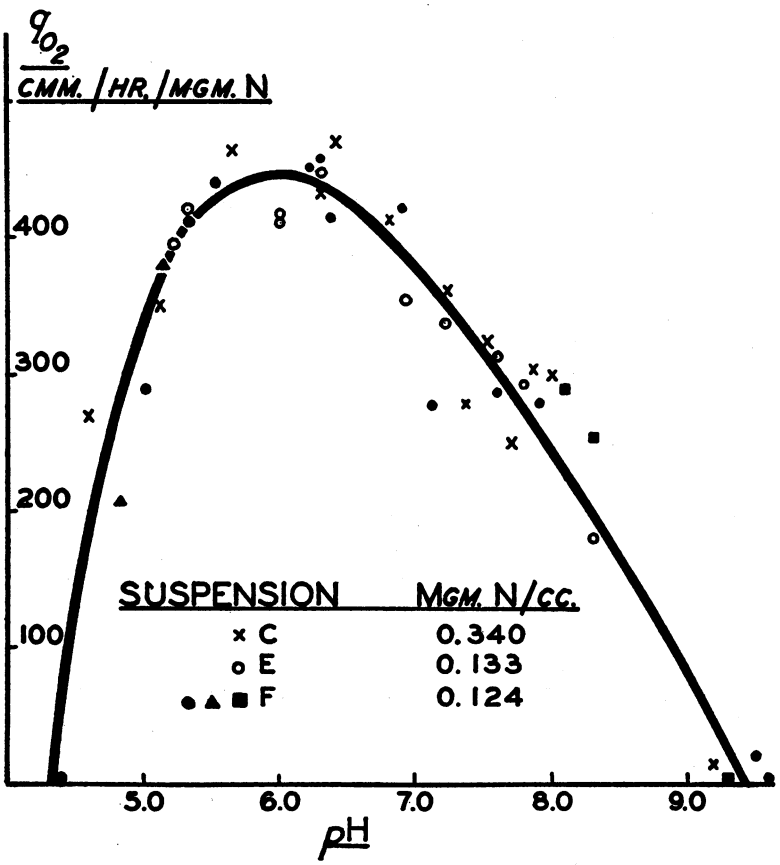

FIG. 4. RATE OF RESPIRATION OF R. TRIFOLII AS A FUNCTION OF pH
Buffer: × ○ ● KH_2PO_4-K_2HPO_4
▲ KH_2PO_4-KHphthalate
■ K_2HPO_4-borate

concentrations did not buffer sufficiently, all measurements were made in a solution of M/30 phosphates.

Effect of reaction. The pH function of the respiration of *R. trifolii* is shown in figure 4 obtained with three suspensions

whose density of cells, as measured by the nitrogen content, varied three-fold. The q_{O_2} values were so constant for the different suspensions that it was possible to plot in one figure all the data without adjustment.

In the region of the neutral point little change in the pH of the suspension plus buffer and substrate occurred during a test. In the extreme acid and alkaline ranges, however, the initial pH differed from the final by as much as 0.4 pH units, doubtlessly due to the low buffering capacity of the mixed phosphates in these regions. This change during a measurement necessitated use of the average value of the pH; hence, these points may not be too accurate. Addition of potassium acid phthalate or sodium borate to the phosphate buffer was shown to be without influence on the respiration. Accordingly, one test was made in which a KH_2PO_4 potassium-acid-phthalate buffer was used in the acid range and K_2HPO_4 borate was used in the alkaline. These buffers were quite stable, and as can be seen from the figure, the respiration values checked those obtained with the mixed phosphates which were open to suspicion.

The optimum pH for respiration of *R. trifolii* is in the range of pH 5.1 to 7.0; there is no well-defined maximum. On either side of the maximum range the rate of respiration decreases rapidly with limits of approximately pH 4.0 and of 9.0. Thorne and Walker (1935) found that the optimum pH for *growth*, as measured by increase in respiration, of *R. meliloti* (alfalfa group) and *R. japonicum* (soybean group) is 7.0. The rate of growth decreased rapidly on either side of the optimum to the limits for growth of pH 4.5 and 9.5. The data of Virtanen and v. Hausen (1931) indicate that the pH optimum for growth of *R. trifolii* on nutrient gelatin extends from 6.5 to 7.5 and that the optimum for fixation of atmospheric nitrogen by nodulated red clover plants is from 6.5 to 7.0 with limits at pH 4.0 and 8.5. Apparently, the optimum reaction for respiration by resting cells of *R. trifolii* is somewhat more acid than the previously observed values for growth of the various *Rhizobium* species. The pH-respiration function of the free-living nitrogen fixer, *Azotobacter*, exhibits a maximum at 7.2 to 7.5 (Burk, Lineweaver and Horner, 1934).

Effect of temperature. The optimum temperature for growth of rhizobia is 28° to 30°C. according to a number of investigators (Fred, Baldwin and McCoy, 1932). As shown in figure 5, however, the optimum for respiration is about 37.5°C. with little change in the rate of respiration throughout the range 35° to 39°C. Because of this rather unexpected difference in the temperature optima for growth and respiration, the effect of temperature on

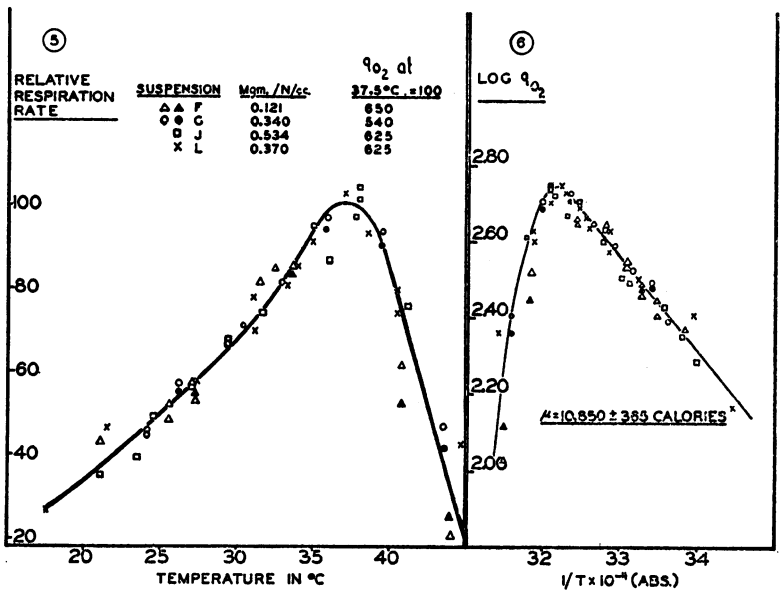

FIG. 5. INFLUENCE OF TEMPERATURE ON RATE OF RESPIRATION OF R. TRIFOLII ○ △ = fresh culture from ice-box. ● ▲ = culture used in previous determination.

FIG. 6. TEMPERATURE CHARACTERISTIC OF RESPIRATION OF R. TRIFOLII

respiration was determined with four different suspensions. As the q_{O_2} for the different suspensions varied to some extent, all were adjusted to a comparable basis by equating the q_{O_2} at 37.5°C. to 100 and calculating the relative values for the other temperatures used. At each temperature the rate of respiration was determined in duplicate. In some instances, at the conclusion of a measurement one of the duplicates was retained, and at the next temperature used, its respiration compared with that

of a fresh culture from the stock suspension kept in the refrigerator. No consistent differences between such duplicates, *i.e.*, fresh and "old," were observed.

When the logarithms of the q_{O_2} values at each temperature are plotted against the reciprocal of this temperature measured on the absolute scale, the points fall along a straight line. The slope of this line multiplied by 4.6 is equal to the Arrhenius temperature characteristic,

$$\mu = R[T_2T_1/(T_1 - T_2)] \log r_2/r_1$$

in which R is the gas constant, 1.99 cal.; r_1, the rate of respiration at the absolute temperature T_1; r_2, the rate of respiration at the absolute temperature T_2. The mean value of μ for the four suspensions is 10,850 $\pm$365 cal. In figure 6 is shown the fit of the points to a line with a slope corresponding to this value of μ after the individual values for each suspension had been adjusted to a common basis. The observed μ is within experimental error of the value, 11,000 calories which Crozier (1924) found to be typical for a large number of biological oxidation reactions. The value of μ for *Azotobacter*, 19,330 $\pm$165 cal., is considerably higher, and the optimum for this organism, 34.5° to 35°C., is definitely lower (Lineweaver, Burk and Horner, 1932).

Effect of concentration of substrate. As indicated in figure 8, the *initial rate of respiration* of glucose by *R. trifolii* is independent of the molality over a hundred-fold range, M/10 to M/1000. With concentrations less than M/100 the rate of respiration decreases with time. Under these circumstances, it is difficult to estimate accurately the *initial rate of respiration*; this probably accounts for the slightly lower value found for M/1000 glucose. When low concentrations of the substrate were used, the respiration practically ceased with an oxygen uptake corresponding to 60 per cent of the glucose present. A similar observation made by Cook and Stephenson (1928) for oxidation of glucose, lactate and acetate by *Escherichia coli* has been recently explained by Clifton (1937). Clifton showed that part of the acetate was *assimilated* by the cells, but if assimilation was prevented by addition of low concentrations of sodium azide or dinitrophenol,

oxidation proceeded to completion. It is likely that part of the glucose is incompletely oxidized to gum by the root nodule organisms.

Technique for making measurements. On the basis of these determinations of the optimum environmental conditions for respiration of *R. trifolii* with glucose as the substrate, the following standard procedure was adopted. To each Warburg flask is added 1 cc. of the suspension of organisms, 1 cc. of M/30

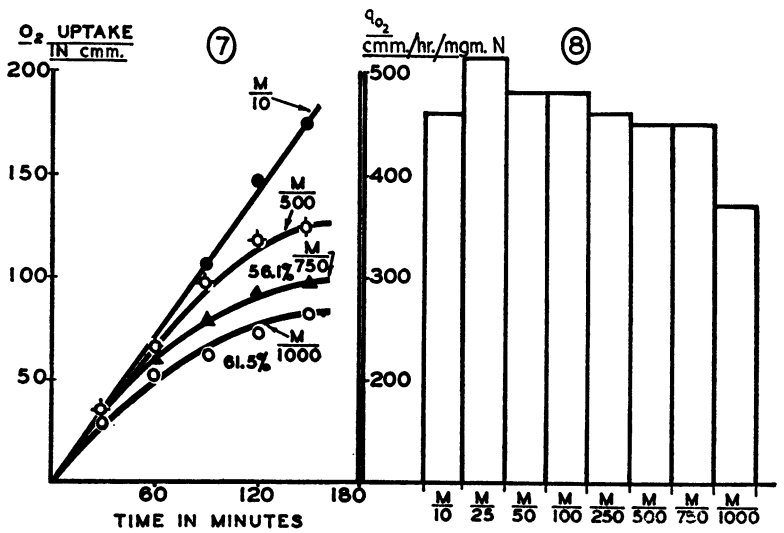

FIG. 7. CURVES OF OXYGEN UPTAKE BY R. TRIFOLII

One cubic centimeter used of each concentration of glucose indicated. Curves for concentrations to M/250 coincide with that shown for M/10. Curve for M/250 begins to drop away from this curve after two hours.

FIG. 8. EFFECT OF CONCENTRATION OF GLUCOSE ON THE INITIAL RATE OF RESPIRATION OF R. TRIFOLII

mixture of phosphates (pH 6.2) and 1 cc. of M/50 substrate. The temperature of the bath is maintained at 34°C. With temperatures near 40°C. a decrease in the rate of respiration with time was often observed. The rate of respiration is near optimum at 34°C., and this temperature is sufficiently low so that inactivation of enzyme through prolonged exposure to the higher temperatures is prevented. If other substances, as inhibitors, are to be added, variations in the foregoing procedure may be made in order to

maintain the total volume of liquid at 3 cc. exclusive of the KOH used to absorb liberated CO_2.

COMPARISON OF RESPIRATION OF RHIZOBIUM TRIFOLII ON DIFFERENT SUBSTRATES

Application of the methods developed was made through a study of the rate of oxidation and the rate of dehydrogenation of methylene blue by *R. trifolii* on different types of substrates. Comparison of the results was facilitated by calculation of these on a relative basis with glucose as the standard, equal to 100. The data are summarized in table 4. The chief conclusions drawn from this study are:

1. General considerations. The relative rate of reduction of methylene blue by the organism in the presence of a given substrate is in general definitely lower than is the rate of oxidation of the same substrate. The *rank*, however, of the substrates as hydrogen donators shows good agreement whether molecular oxygen or methylene blue is taken as the hydrogen acceptor. Exceptions to this generalization are: arabinose and galactose are excellent hydrogen donators to O_2 but only fair to methylene blue; the reverse is true of formate; acetate is a very poor donator to methylene blue but is fair to good with oxygen.

2. Carbohydrates. No carbohydrate examined was as good a hydrogen donator as is glucose with the possible exception of arabinose. Glucose, fructose, galactose, mannose, arabinose, sucrose and maltose are good to excellent hydrogen donators to O_2 in the presence of *R. trifolii*; xylose, lactose and cellibiose are fair; rhamnose and raffinose are poor.

3. Polyhydric alcohols. The polyhydric alcohols were oxidized by O_2 in an unusual manner. The initial rate of respiration, which was rather low, increased with time; with carbohydrates the rate of respiration is constant. The rates for the first and last 45 minutes of the 2 to 3 hours' tests are given in the table. The initial lag suggests the formation of some intermediate, possibly the corresponding aldose, which provides a better substrate for respiration. The consistency of the results, independent of the number of carbon atoms in the polyhydric

TABLE 4

Relative rates of dehydrogenation of different sources of carbon by suspensions of R. trifolii using oxygen and methylene blue as hydrogen acceptors

SUBSTRATE	SUSPENSION 6	SUSPENSION 7		SUSPENSION H		AVERAGE	
	M.B.	M.B.	O_2	O_2	O_2	M.B.	O_2
I. Carbohydrate							
Glucose	100	100	100	100	100	100	100
Fructose	69	55	86	76	79	62	72
Galactose	51	56	87	98	96	54	94
Mannose		65	100			65	100
Xylose	53	64	67	63	66	58	65
Arabinose	57	63	116	118	104	60	112
Sucrose		62	85	90	85	62	87
Maltose	61	72	80		81	66	81
Lactose	28	28	51	62	66	28	60
Rhamnose				29	21		25
Cellibiose				73	67		70
Raffinose				33	18		25
II. Alcohols							
Glycerol					47 73		47 73
Erythritol	17	16	54 66	29 53	28 60	17	37 60
Sorbitol	49	60	77 90			55	77 90
Mannitol	31	29	56 94	32 77	38 79	30	42 83
III. Acids							
Formate	85	95	41	38	22	90	34
Acetate	19	0	51 81	42 67	40 64	10	44 71
Lactate	83	76	87	83	50	80	73
Pyruvate	32	37	72	76	74	35	74
Succinate	95	87	183	147	140	91	157
Malate	90	90	108			90	108
Fumarate	113	96	173	130	122	105	142
Maleate				27	25		26
Citrate	0	0	27	24	15	0	22
Tartrate	0	0	27	20	12	0	20
Oxalate				19	15		17

When two values are indicated, the first is the initial rate of respiration, the second is the final. All other values indicate that rate of respiration was constant throughout test.

alcohol, indicates a common mechanism for oxidation of this type of compound. With O_2 as the hydrogen acceptor there was little difference in the activity of these compounds; with methylene blue all were poor with the possible exception of sorbitol.

4. Acids. The oustanding feature of the studies on substrates was the rapid utilization of the four carbon dicarboxylic acids. With O_2 as the acceptor, the highest rates of respiration observed with any substrate were obtained with fumarate and succinate. Malate, although not quite as active as these two, was also an excellent hydrogen donator to O_2. With methylene blue these compounds were not so outstanding as hydrogen donators but were superior to the majority of substrates tested. Since these three acids together with oxaloacetic acid act as catalysts in respiration of certain tissues (Annau, *et al.*, 1935; Laki, Straub, and Szent-Györgi, 1937), and since oxaloacetic acid may be concerned with the symbiotic nitrogen fixation process (Virtanen and Laine, 1937), respiration of the rhizobia with these acids as substrates is being investigated in detail.

Respiration on formate was very poor and fell off rapidly with time, but this compound was one of the best donators to methylene blue. In contrast, acetate was poor to inactive with methylene blue as the acceptor but fairly active with O_2. Oxygen uptake with acetate was of the same type as with the polyhydric alcohols, viz., increase in the rate with time suggestive of the formation of an intermediate which is more readily attacked. Lactate and pyruvate were both fairly good hydrogen donators to O_2, but pyruvate was poor with methylene blue as the acceptor. It is likely that lactate is readily dehydrogenated to pyruvate in the presence of methylene blue, but further activation of hydrogen proceeds much more slowly. Citrate, tartrate, maleate, and oxalate were very poor hydrogen donators to O_2 and inactive toward methylene blue.

SUMMARY

The preparation of a suspension of "resting cells" of *Rhizobium trifolii* which has suitable characteristics for study of the respiratory activities of this organism is described.

The influence of storage of the cells, dilution of the suspension, addition of the specific stimulatory factor ("Coenzyme R"), concentration of buffer salts, pH, temperature and concentration of substrate on the respiration of *R. trifolii* has been determined. From these investigations has been developed a suitable technique for the study of the respiratory characteristics of this organism.

Comparison of the relative activity of a number of carbohydrates, polyhydric alcohols and organic acids as hydrogen donators when activated by *R. trifolii* with O_2 or methylene blue as acceptors has been made.

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