A SIMPLE METHOD FOR MAKING MULTIPLE TESTS OF A MICROORGANISM

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In applying different biochemical reactions to microorganisms, considerable time and expense can be saved by the following method, which makes it possible to make at least 16 tests on a single plate of either the primary growth or a transplant of it.

Press sterile glass tubes, 10 mm long and 9 mm O.D., aseptically about 1 mm into the surface of a plate of the selected culture medium, put one or two drops of the appropriately diluted reagents into the resulting cups, and observe any changes.

The method is capable of numerous applications. For example, in determining reactions of staphylococci, the 48 hour growth on staphylococcus medium no. 110 (J. Bact., **51,** 409, 1946) is "cupped," one or two drops of 1:10 of the "indicator" dilution of either chlorphenol red, bromcresol purple, bromthymol blue, or phenol red are added to determine fermentation of mannitol. Reduction can be determined by using triphenyl tetrazolium chloride and observing reduction in about 5 minutes. Gelatinolysis can be determined by putting 20 per cent sulfosalicylic acid into a 22 mm O.D. "cup" around an isolated colony.

PHOTOHYDROGEN PRODUCTION IN CHROMATIUM[1]

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Previous observations on the photochemical production of molecular hydrogen during metabolism of various species of *Athiorhodaceae* have been extended to *Chromatium* sp., a representative of the *Thiorhodaceae.*

Stab cultures were maintained under constant illumination in media compounded, according to a prescription kindly supplied us by Dr. J. L. Stokes, Indiana University, Bloomington, Indiana, as follows: 100 ml tapwater, 100 mg NH_4Cl, 100 mg KH_2PO_4, 50 mg $MgCl_2$, 3.0 g NaCl, 100 mg yeast extract (Difco), and 2.0 g agar. After sterilization, 4 ml 5 per cent $NaHCO_3$ and 2 ml 5

[1] This note is the thirteenth in a series of publications dealing with the metabolism of photosynthetic bacteria. For a complete list of references consult Arch. Biochem. and Biophys., *in press,* (1952).

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per cent $Na_2S \cdot 9H_2O$ solution (sterilized separately) were added, and the pH adjusted to 8.0 to 8.5. Stabs were sealed with a 1:1 mixture of paraffin and paraffin oil.

For growth experiments a basal medium of the following composition was prepared: 100 ml tapwater, 100 mg KH_2PO_4, 50 mg $MgCl_2$, 3 g NaCl, 300 mg L-glutamic acid, and 100 mg yeast extract. The hydrogen donors used were DL-malic acid, Na_2S, and $Na_2S_2O_3$. In the case of malate, 300 mg were added per 100 ml basal medium, the resultant solution then being neutralized and autoclaved. When either of the sulfur compounds was used, the basal medium was first neutralized and autoclaved, then sterile solutions of the sulfur compounds were added in the following amounts: per 100 ml basal medium, 2 ml 5 per cent $Na_2S \cdot 9H_2O$, or 1 ml 1.0 M $Na_2S_2O_3$ together with 0.4 ml 5 per cent $Na_2S \cdot 9H_2O$. Sulfide was included in all media to ensure absence of oxygen. Glass-stoppered bottles completely filled with appropriate media were inoculated with a suspension, in sterile 3 per cent NaCl solution, of oxygen-free organisms obtained from a stab culture. Liquid cultures were grown in a constant temperature bath (25 to 30 C) under constant illumination with a bank of Mazda bulbs. Equal inocula were used in any given experiment. Bottles were allowed to stand overnight before inoculation to ensure complete removal of oxygen. In some experiments, cell suspensions were grown anaerobically in fermentation tubes.

Good growth was observed, concomitant with profuse gas production, when the medium contained malate with or without one or the other of the sulfur compounds. By using no more than 1 ml 0.1 M $Na_2S_2O_3$ per 100 ml basal medium containing 300 mg DL-malic acid, good growth and gas production were observed without noticeable storage of sulfur. If more (i.e., 1.0 ml of 1 M $Na_2S_2O_3$) was used, considerable sulfur storage occurred, the cultures becoming whitish in appearance.

All experiments on "resting cells" were conducted using Warburg vessels with 0.2 ml 10 per cent KOH absorbed on filter paper in the center well. Substrates were added from a sidearm in such amounts that their final concentrations were closely equal to those used in growth experiments. The main compartment contained 1.8 ml bacterial suspension, 1 mg $MgCl_2$ (0.1 ml standard $MgCl_2$ solution), and 0.1 ml 5 per cent $NaHCO_3$. The suspending medium was either oxygen-free saline phosphate buffer (composition: 100 ml H_2O, 880 mg K_2HPO_4, 3 g NaCl, 1 to 2 drops 1 N HCl, with optional addition of 1.0 ml 5 per cent $Na_2S \cdot 9H_2O$) or saline borate—HCl buffer (Umbreit, Burris, and Stauffer: Manometric Techniques and Tissue Respiration, Burgess, 1949, p. 133).

The organisms were washed once or twice with 20 ml portions of buffer and then suspended in 10 ml buffer. The final cell densities were approximately 25 to 40 μl wet volume per ml suspension.

Dark experiments were conducted as usual by wrapping test vessels in aluminum foil. Because of the alkalinity of the media, any gas production could be taken as preliminary evidence for hydrogen production. This was confirmed in all cases by the ignition test.

Using only inorganic media, no gas production could be observed even over

periods as prolonged as 2 to 3 weeks. Whenever malate media were used, good gas production, as well as growth, was observed in 2 to 3 days. Attempts to establish production of hydrogen by resting cells suspended in strictly inorganic media failed, primarily because of occurrence of high variable endogenous effects. Uniformly positive results with resting cells were obtained only when malate was supplied as hydrogen donor. No gas production was observed in dark controls. The inhibitory effect of ammonium ion or nitrogen gas, peculiar to photohydrogen production in *Athiorhodaceae* (Gest, Kamen, and Bregoff: J. Biol. Chem., **182**, 153, 1950), was also observed with *Chromatium*.

TABLE 1

Photohydrogen production in resting cell suspensions of Chromatium

Experimental conditions: main compartment (see text). Zero time, 0.1 ml (= 37.2 μM) Na malate, 0.1 ml H_2O, or 0.1 ml (= 20 μM) Na thiosulfate added. Center well, 0.2 ml 10 per cent KOH. Temp, 30 C; gas phase, He.

EXPERIMENT	pH	TIMES WASHED	CELL DENSITY	DURATION	ENDOGENOUS* + MALATE* + THIOSULFATE*		
			µl/ml	*hr*			
1	8.0	once	30	7½	22.8	32.7	—
2	8.0	twice	38	6½	6.5	19.0	—
3	8.7	twice	28	7	17.2	24.6	18.0

* Values denote micromoles H_2 evolved.

In table 1, typical results obtained with *Chromatium* suspensions in borate buffer at pH 8.0 and 8.7 are exhibited. It is seen that while the addition of malate resulted in a definite stimulation of hydrogen production over that obtained with endogenous controls, no such effect could be observed with thiosulfate. Attempts to reduce endogenous hydrogen production by exhaustive washing were frustrated by the extreme fragility of the suspensions. It was found that, despite every care, two successive washes usually resulted in marked reduction of gas metabolism, with or without added substrates. Results following one or two washes are included in table 1.

Attempts to observe photohydrogen production in inorganic media will be resumed if a suitable organism can be found among the purple or green sulfur bacteria.

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