A CHEMICALLY DEFINED MEDIUM FOR GROWTH, TRANSFORMATION, AND ISOLATION OF NUTRITIONAL MUTANTS OF HEMOPHILUS INFLUENZAE[1]

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Received February 23, 1960

The field of genetic transformation in bacteria has long needed defined media in which the separate steps of the total process could be analyzed. Hemophilus para-influenzae (Herbst, et al., 1955), Diplococcus pneumoniae (Rappaport and Guild, 1959), and Bacillus subtilis have been grown in defined media, but apparently only B. subtilis (Spizizen, 1959) was then transformable.

Since the classical experiments of the Lwoffs (1937) it has been known that H. influenzae is dependent for growth on hemin or its homologues and on pyridine nucleotide coenzymes of which diphosphopyridine nucleotide (DPN) is most frequently used. Obtaining a defined medium for the rough form of type d H. influenzae began with the finding that the tissue culture medium M-199 (Morgan, et al., 1950) and a selected group of supplements supported growth of this organism both in liquid medium and on agar plates[3]. A 10-100 cell inoculum of H. influenzae resistant to several antibiotics was grown to a level of 10^8-10^9/ml and then diluted back to 10-100 cells per inoculum and the cycle repeated eight times. Proof that the final culture was composed largely of decendents of

[1]Supported in part by Public Health Service Research Grant E-1218, and Atomic Energy Commission contract AT(30-1)-1371.

[2]Supported by Public Health Service Training Grant 2G-73, Division of General Medical Sciences.

[3]0.04 M phosphate buffer made up of two parts dibasic to one monobasic was necessary to raise the 2% agar to pH 7.5. Equal parts of this were mixed with twice concentrated synthetic medium.

the initial inoculum was obtained by isolation of the same antibiotic resistance transforming factors and by the obligate dependence of the cells on DPN and hemin.

Elimination of unnecessary constituents from the medium led to a composition shown in Table I. These constituents are of four types: (a) essential and not replaceable, of which arginine is the only instance; (b) critical but replaceable by related substances; when this type is omitted, 10^6 cells/ml fail to grow to 10^8 cells/ml in five days

TABLE I

Synthetic Growth Medium for Hemophilus Influenzae

Substance (in order of mixing°)	Type* Substance	Milligrams/Liter	Micromoles/Liter
NaCl	d	5.7×10^3	9.7×10^4
KCl	d	330	4.4×10^3
$MgSO_4 \cdot 7H_2O$	d	180	730
$NaH_2PO_4 \cdot 2H_2O$	d	110	700
Glucose	b	4200	2.3×10^4
$CaCl_2$	d	180	1600
Thiamine HCl	c	8.4	24
DPN	b	8.4	12
Na Acetate	d	42	512
Uridine	b	8.4	35
Biotin	c	8.4	35
Choline chloride	c	8.4	60
Thymidine	c	8.4	34
Inositol	c	8.4	38
Ca Pantothenate	b	8.4	17
Desoxyadenosine	c	17	62
Pyridoxine HCl	c	8.4	40
L-Tyrosine (HCl)	b	75.5	405
L-Cystine (HCl)	b	42	175
L-Arginine (HCl)	a	135.2	782
L-Glutamic acid	c	176.4	1180
L-Leucine	c	303	2310
Desoxyguanosine	b	8.4	29
L-Tryptophane	c	57.8	284
Glutamine	b	252	1800
Hemin + Histidine	b + d	8.4 (each)	Hemin = 12 Histidine = 54
Triethanolamine	d	.37	2.4
Polyvinyl alcohol	d	840	
Glycylglycine	d	1580	1.0×10^4
$NaHCO_3$	d	225	2.6×10^3
Tween 80	d	20	

°This order of mixing prevents precipitation.

*See text for an explanation of this key.

at 37°C; (c) stimulatory--omission reduces the growth rate; and (d) inorganic salts, buffers, surface active agent, protective colloid, and solubilizing aids.

The generation time of the organism in the defined medium is close to one hour, and colonies appear after 36 hours incubation on plates made up of agar and synthetic medium. Both results suggest that the growth rate is about half that observed in the more complex Levinthal-Eugonbroth medium (Goodgal and Herriott, 1960).

Having the above defined medium, nutritional mutants are readily obtained. Cells exposed to ultraviolet irradiation until only 0.1 per cent of the original number survived (ca. 7 lethal doses per cell) increased the number of mutants, which after an initial short period for phenotypic development in broth medium were selected by either of two different techniques. In one, the cells were plated in synthetic medium in which the glucose was replaced by another simple carbohydrate. In this way mutants utilizing mannose, mannitol or sorbitol were obtained. Auxotrophic mutants were selected by a modification of the penicillin technique of Lederberg and Davis (1950). Following irradiation an inoculum was introduced into synthetic medium containing penicillin at 500 units per ml. After an overnight incubation the cells were washed and placed in fresh synthetic medium containing an additional nutrient. In this way three mutant strains were derived requiring respectively lipoic acid, deoxycytidine, and l-methionine, in addition to the basal medium of Table I.

Transformations of cells grown in the synthetic medium have been obtained, but the frequency was very low. Addition of 10 μg/ml of agmatine to the growth medium raised the frequency to one in five thousand, which is still only one per cent of the figure obtained with cells grown in rich Levinthal media. Highly competent cells prepared in Levinthal media lose their competence on repeated washing with buffered

saline. At least an 80 per cent recovery of competence is obtained on resuspending the cells in the synthetic medium, showing that an extrinsic factor(s) essential for competence is present among the known constituents.

A mannose-utilizing mutant yielded DNA that transformed wild-type cells from negative to positive mannose utilization. Similar tests on the other mutants are in progress.

H. aegyptius grows readily in the described medium, as does para-influenzae with the addition of putrescine, but H. influenzae suis does not grow suggesting that other nutrients may be needed. Knowledge of these additional nutrients for the suis strain should provide a new basis for studies of the unique synergistic action of this organism with the virus of swine influenza (Shope, 1931).

While the development of the defined medium was undertaken to permit a better understanding of the mechanism of genetic transformation, it also provides a means of studying a number of interesting metabolic pathways, because this organism and its mutants have such unique nutritional requirements.

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