THE INFLUENCE OF SODIUM ACETATE UPON THE DISSOCIATION OF A STRAIN OF HEMOLYTIC STREPTOCOCCUS

ALICE P. McILROY, A. E. AXELROD, AND RALPH R. MELLON[1]

Institute of Pathology of the Western Pennsylvania Hospital and the Department of Chemistry of the University of Pittsburgh, Pittsburgh, Pennsylvania

Received for publication July 24, 1948

It is generally recognized that a variety of environmental factors may influence the process of bacterial dissociation. Inasmuch as most of these studies have been conducted with media composed of crude natural materials, it was considered advisable to employ media of known chemical constitution in order to determine more precisely the nutritional factors capable of implementing this variation phenomenon. The inquiry is particularly pertinent in view of the current tendency to explain dissociative behavior, as well as other categories of variation, on a mutation basis. According to Braun (1947), the chief influence of environment is to select out the spontaneously occurring mutants.

The present paper is the first of a series dealing with the use of such "synthetic" media in studies upon bacterial dissociation. The term "synthetic" as used in this work designates media in which a hydrolyzate from purified casein is the only substance the chemical constitution of which is not absolutely established. This study is concerned with the role of sodium acetate in the maintenance of the mucoid character and virulence of a strain of hemolytic streptococcus.

METHODS

Culture. A culture of hemolytic streptococcus, Lancefield group C, Stoddard strain (Mellon and Cooper, 1938), was used throughout the investigation. This culture is mucoid in character and has a high virulence for white mice (MLD = 2 to 6 organisms). As a stock culture, it has been carried in semisolid veal heart infusion agar (0.2 per cent) containing 5 per cent defibrinated rabbit's blood. This medium is adjusted to pH 7.6. The culture has been passed through mice at frequent intervals in order to maintain its virulence.

Determination of virulence. Virulence tests were conducted by preparing varying dilutions of actively growing cultures in veal heart infusion broth and injecting 0.5-ml quantities intraperitoneally into white mice weighing 20 to 25 grams. Each dilution was administered to three mice and the animals were observed for ten days. The MLD was taken as the minimum number of organisms necessary to cause death in two of the three animals in each group.

Special media. The three synthetic media employed are shown in table 1. All experiments were conducted with 5 ml of single strength medium per culture tube.

[1] We are indebted to Lederle Laboratories, Incorporated, for the synthetic folic acid, and to Merck and Company for the pyridoxal hydrochloride and the pyridoxamine dihydrochloride.

TABLE 1

*Constituents of media**

MEDIUM A†			MEDIUM B		
Constituents	Concentration‡		Constituents	Concentration‡	
Casein hydrolyzate§	100	ml	Casein hydrolyzate§	100	ml
KCl	6	g	Sodium acetate·$3H_2O$	40	g
$Na_2HPO_4 \cdot 12H_2O$	2	g	Sodium citrate·$2H_2O$	1	g
KH_2PO_4	5	g	Glucose	40	g
$MgSO_4 \cdot 7H_2O$	1	g	K_2HPO_4	1	g
Glucose	66.5	g	KH_2PO_4	1	g
L-Cystine	270	mg	L-Cystine	200	mg
Uracil	20	mg	L-Asparagine	200	mg
Adenine	20	mg	Guanine	20	mg
Nicotinic acid	2	mg	Uracil	20	mg
Pyridoxine hydrochloride	2	mg	Adenine	20	mg
Calcium pantothenate	8	mg	Nicotinic acid	2	mg
Thiamine hydrochloride	2	mg	Pyridoxine hydrochloride	2	mg
Riboflavin	1	mg	Calcium pantothenate	8	mg
L-Tryptophan	40	mg	Thiamine hydrochloride	2	mg
$CuSO_4 \cdot 5H_2O$	2	mg	Riboflavin	1	mg
$ZnSO_4 \cdot 7H_2O$	2	mg	L-Tryptophan	40	mg
$FeSO_4 \cdot 7H_2O$	2	mg	Pyridoxal hydrochloride	2	mg
$MnCl_2 \cdot 2H_2O$	0.8	mg	Pyridoxamine dihydrochloride	2	mg
$CaCl_2 \cdot 2H_2O$	20	mg	Xanthine	20	mg
Biotin	0.0004	mg	*p*-Aminobenzoic acid	0.1	mg
Adjust to pH 6.5‖ and autoclave			*i*-Inositol	1	mg
			$MgSO_4 \cdot 7H_2O$	400	mg
			$FeSO_4 \cdot 7H_2O$	20	mg
			$MnSO_4 \cdot 2H_2O$	12	mg
			NaCl	20	mg
$NaHCO_3$¶	4	g	$CuSO_4 \cdot 5H_2O$	2	mg
L-Glutamine¶	400	mg	$ZnSO_4 \cdot 7H_2O$	2	mg
Thioglycolic acid¶	260	mg	$CaCl_2 \cdot 2H_2O$	20	mg
			Folic acid	0.001	mg
			Biotin	0.0004	mg
			Adjust to pH 6.5‖ and autoclave		
			$NaHCO_3$¶	4	g
			L-Glutamine¶	400	mg
			Thioglycolic acid¶	260	mg
Final pH	7.5		Final pH	7.5	

* Medium C was prepared by omitting the following compounds from Medium B:—sodium citrate, L-asparagine, guanine, pyridoxal hydrochloride, pyridoxamine dihydrochloride, xanthine, *p*-aminobenzoic acid, *i*-inositol, and folic acid.

† Medium A is essentially that of Bernheimer *et al.* (1942).

‡ Concentration per liter of double strength medium.

§ Casein hydrolyzate, 10 per cent, "vitamin-free," acid-hydrolyzed (SMACO).

‖ The pH was adjusted with 10 per cent sodium hydroxide solution.

¶ Added aseptically to the autoclaved medium.

The glutamine solution was sterilized by filtration through a sintered glass filter. Sodium bicarbonate and thioglycolic acid were sterilized as described by Adams and Roe (1945).

Inoculation procedures. For each experiment a stock culture of maximum virulence was plated on blood agar and incubated 18 to 20 hours at 37 C. A tube of the synthetic medium (table 1) under investigation was inoculated with this culture. After incubation at 37 C for 18 hours, the cells were separated by centrifugation, washed with normal saline, and resuspended in 5 ml of saline. This suspension (0.1 ml) was used to inoculate the original synthetic medium or any modification thereof. After incubation for 4 to 5 hours at 37 C, the growth and pH measurements were made on duplicate cultures. The amount of growth was determined turbidimetrically with an Evelyn photocolorimeter using a 660 filter, and by the plate count method. The pH measurements were made with a Beckman pH meter. After storage overnight at 5 C, 0.2 ml of the culture were used to inoculate a fresh batch of the same medium. This procedure was repeated for every daily serial transfer. Virulence tests were performed on cultures from the final transfer.

In the early experiments, an 18-hour incubation period was employed in the serial transfers. The results obtained with this procedure did not differ from those obtained when the shorter incubation periods were used. The latter procedure has the advantage of avoiding effects due to the lowered pH and to the accumulation of metabolic products found in aging cultures.

EXPERIMENTAL RESULTS

In the original experiments employing synthetic medium A (table 1) a decrease in mucoid character and mouse virulence was noted after daily, serial transfers. Since this culture maintained both of these characteristics on a veal heart infusion blood medium, it is apparent that a constituent (or constituents) present in the natural medium and missing from the synthetic medium A was essential. Experiments to determine the nature of this essential factor (or factors) were initiated.

Our first efforts were directed toward an attempt to prepare a synthetic medium that was comparable to the natural medium in its ability to maintain mucoidness and virulence. It was hoped that a systematic elimination of various components of such a complete synthetic medium would lead to the identification of the essential factor(s). Accordingly, medium A was supplemented with a variety of compounds to yield medium B (table 1). This medium was capable of maintaining the mucoid character and virulence on daily, serial transfer. A systematic elimination of the various components of medium B was undertaken, and it was found that the omission of sodium acetate resulted in a medium which permitted the mucoid to smooth transformation. Sodium acetate was specific in this regard. It should be noted that the original medium employed (medium A) did not contain sodium acetate, thus explaining its inability to maintain the mucoid character.

The omission of the following compounds from medium B affected neither growth nor the ability to maintain a mucoid state: sodium citrate, L-asparagine, guanine, pyridoxal hydrochloride, pyridoxamine dihydrochloride, xanthine, *p*-aminobenzoic acid, *i*-inositol, and folic acid. To simplify our procedure,

these constituents were omitted from medium B to yield medium C (table 1). All of the subsequent results reported in this paper were performed with medium C, or with medium C lacking sodium acetate.

On the third to fourth transfer in the medium lacking acetate, transformation from the mucoid into the smooth phase became evident. At the sixth transfer only the smooth phase was present. As the cultural characteristics changed, the morphology of the organism also underwent change. The original mucoid culture composed of encapsulated cocci in very short chains dissociated to a long-chained streptococcus with loss of capsular material. Identical results were obtained in ten experiments. No evidence of any change from the mucoid phase has been noted after 64 daily, serial transfers in either medium C (containing acetate) or in veal heart infusion blood broth.

The virulence of the smooth phase for mice was considerably less than that of the mucoid phase. Thus the MLD of the mucoid phase (grown in veal heart infusion blood broth or in medium C) and the smooth phase (grown in medium C minus acetate) was 5 and 5,000 organisms, respectively.

The stability of the smooth phase was investigated. When subcultured in medium C, no transformation into the mucoid phase was noted after 14 daily, serial transfers. The decreased mouse virulence of the smooth phase was also unaffected by this procedure. In contrast, when passed serially through mice in numbers large enough to cause death, a pure culture of the smooth phase gradually reverted to the mucoid phase. At the tenth serial transfer, only the mucoid phase was evident. The virulence of this culture was identical with that of the original mucoid organism.

As determined both by the plate count and turbidimetric measurements, the omission of acetate from medium C was without effect upon the extent of growth of the mucoid phase during the first few serial transfers. After continued subculture, transformation into the smooth phase occurred. The growth rates of this resulting smooth phase in both acetate-free and acetate-containing media were identical and equal to that of the original mucoid culture. The pH of cultures grown both in the presence and absence of acetate never fell below 7.0. To further eliminate the possibility that the buffering action of acetate was significant in the maintenance of the mucoid phase, cultures were serially transferred in media in which the acetate was substituted by phosphate, citrate, and bicarbonate buffers. The mucoid phase could not be maintained in the presence of these buffers, thus demonstrating that the activity of acetate was not due to its buffering capacity.

DISCUSSION

The application of "synthetic" media to studies upon bacterial dissociation possesses a twofold advantage. First, the reproducibility of the medium can be maintained within rather narrow limits. This is of particular importance since there is formidable evidence that variations in environment can markedly influence the process of bacterial dissociation. The extreme consistency of the results reported here bears witness to the reproducibility of our environmental

conditions. The past experience of this laboratory in studies of this type has been entirely with media composed of crude, natural materials and a not infrequent inability to reproduce certain results has been noted. It is conceivable that variations in the composition of the natural materials contained in these media could account for some of these anomalous results.

In the second place, a medium of known chemical constitution is essential in studies designed to determine the role of individual nutrilites upon bacterial dissociation. The flexibility of such media in regard to the ease with which the individual components may be varied is evident.

The present experiments have demonstrated that the elimination of sodium acetate from a "synthetic" medium determines the transformation from the mucoid to the smooth growth phase. It should be noted that as long as the mucoid phase is able to grow without acetate, the absence of the latter affects neither the growth rate of the organism nor the final pH of the culture medium. The M to S phase change, therefore, is due primarily to the absence of sodium acetate and is not the result of secondary influences upon rate of growth or degree of acidity. This point requires emphasis since changes in the pH of the medium, and bacteriostatic changes particularly, are well known to affect the incidence of bacterial dissociation. The fact that the M phase is replaced by the S phase in the absence of acetate is not interpreted as a bacteriostatic or bactericidal effect, but rather a change incident to a nutritional deficiency.

With regard to the mechanism of action of the sodium acetate, we have adopted the tentative view that this compound functions not as a growth factor but as a precursor in the biosynthesis of some higher molecular weight compound(s) which is essential—perhaps, as a structural component of the capsular material—for the maintenance of the mucoid state. Apparently an exogenous source of acetate is required for this function. In the absence of added acetate, the transformation to a smooth phase, which is stable even in the presence of acetate, occurs.

The alternate view, namely, that acetate is essential for the growth of the mucoid and not the smooth phase, is difficult to substantiate, since transformation into the smooth phase occurs following continued subculture in the absence of acetate. At least for the first few serial transfers into a medium lacking acetate (before the M to S change is recognizable) no bacteriostatic effect is noted.

The precise nature of the postulated compound into which acetate is presumed to be converted is unknown. There is ample evidence that acetate may serve in the biosynthesis of a variety of tissue constituents, such as glycogen, uric acid, fatty acids, cholesterol, dicarboxylic amino acids, and protoporphyrin (Bloch, 1947). Guirard *et al.* (1946) have reported on the probable role of acetate as a precursor of various lipoidal materials in bacterial metabolism.

Preliminary experiments have indicated that lipids may replace acetate for the maintenance of the mucoid character of our strain of hemolytic streptococcus.

The relationship between mucoid character and virulence is not clear. In the group C organism employed in this study, maximum virulence is characteristic

only of the mucoid state. The conditioning influence of the acetate on the mucoid state in these experiments tends to emphasize the likelihood that mucoid nature and virulence do not represent linked characters, but rather an association of characters. It is interesting to note in this connection that our experience with certain strains of group A streptococci has indicated that an exogenous source of acetate is not necessary for either the growth or the maintenance of mucoid character. Although the majority of group A strains are highly virulent in the mucoid phase, it has long been known that exceptions occur. In the case of group A streptococci, it is well known that virulence is dependent upon the presence of the M antigen, and the presence of this antigen gives rise to the so-called "matt" appearance that is characteristic of the colonies produced by virulent, but nonmucoid, strains of group A streptococci.

The current genetic appraisal of this M to S transformation would probably say that we are dealing with a very low frequency of mutation rate on the part of the mucoid phase, which phase has a suppressive effect on the appearance of the resulting S mutant. This suppressive effect would be abrogated by the absence of acetate, which would select out the occasional mutant S cell by depriving the M cells of a chance to grow.

This interpretation, however, fails to accord with the results of experiments designed to test its validity. Thus two single S phase cells were mixed in acetate-containing broth containing 5,000,000 M phase cells. After 18 hours' incubation at 37 C, equal numbers of M and S forms were present.

Furthermore, after 60 serial transfers of the M phase on blood agar had shown no S phase colonies, 12 isolated M colonies from a single plate were selected at random for test of the frequency of the M to S transformation. Transfers were made from each of six colonies into its own tube of acetate broth; and six colonies were likewise transferred into the same synthetic medium without acetate. After the fifth serial transfer in the latter medium, all six M phase cultures went over to the S phase, with only occasional mucoid colonies appearing. At this time all six M phase cultures in acetate broth remained mucoid, no S colonies appearing.

In other words, there is not the slightest evidence of a suppressive effect of M over S; in fact, the phenomenon of "population dynamics" appears to be in reverse. As to frequency, the transformation occurred in 100 per cent of the colonies tested. Thus, it seems permissible to infer that the burden of proof in this instance is shifted to the opponents of the theory of adaptive modification by specific environments.

SUMMARY

It has been demonstrated that the presence of sodium acetate in a synthetic medium is necessary in order to maintain the mucoid character and virulence of a strain of group C hemolytic streptococcus upon repeated daily subculture. The elimination of acetate permits the transformation from the virulent mucoid phase to a relatively avirulent smooth phase. The smooth phase does not revert to the mucoid state *in vitro* in the presence of acetate, but does completely

revert after ten passages in mice. This phase change has been shown to be due primarily to the absence of sodium acetate and is not the result of secondary influences upon rate of growth or degree of acidity.

All the evidence to date points to the likelihood that the transformation is not the result of a spontaneous mutation, but is an adaptive response to the absence of a nutrilite so vital to the mucoid phase that this phase may be said to have no existence in its absence.

REFERENCES

ADAMS, M. H., AND ROE, A. S. 1945 A partially defined medium for the cultivation of pneumococcus. J. Bact., **49**, 401–409.

BERNHEIMER, A. W., GILLMAN, W., HOTTLE, G. A., AND PAPPENHEIMER, A. M., JR. 1942 An improved medium for the cultivation of hemolytic streptococcus. J. Bact., **43**, 495–498.

BLOCH, K. 1947 The metabolism of acetic acid in animal tissues. Physiol. Revs., **27**, 574–620.

BRAUN, WERNER 1947 Bacterial dissociation. Bact. Revs., **11**, 75–114.

GUIRARD, B. M., SNELL, E. E., AND WILLIAMS, R. J. 1946 The nutritional role of acetate for lactic acid bacteria. Arch. Biochem., **9**, 361–379, 381–386.

MELLON, R. R., AND COOPER, F. 1938 Bi-phasic nature of certain alpha-prime hemolytic streptococci. Proc. Soc. Exptl. Biol. Med., **38**, 158–160.