THE GRANULOSE REACTION OF CERTAIN ANAEROBES OF THE "BUTYRIC" GROUP

ROBB SPALDING SPRAY[1]

Medical School, West Virginia University, Morgantown, West Virginia

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McCoy, Fred, Peterson, and Hastings (1926) state: "One of the most distinctive features of the group to which the butyl organism belongs is the granulose reaction. Young vegetative cells . . . stain yellow with iodine. As sporulation approaches and the cells take on clostridial form, they store granulose and stain blue or violet with iodine. As the spores mature the granulose reaction is lost, indicating that the reserve material has been utilized in sporulation." This microscopic observation has long been used as a criterion for the classification of members of this iodophilic group. Batchelor and Curie (1929) have grown cultures of soil butyrics in oat jars, then flooded the plates with iodine, and determined the iodophiles by the macroscopic observation of the blue-black colonies.

In reporting a study of the cultivation and identification of the butyric anaerobes (Spray, 1937), there was discussed a striking phenomenon observed in semisolid agar cultures of certain members of this group. This reaction was obtained when Gram's iodine was added to glucose semisolid agar cultures, which immediately turned dark violet. This reaction was consistently obtained with only certain members of the group and thus appears to have a possible value for the differentiation of certain species. In view of the chaotic taxonomy of the butyric group, such a "species" character may prove of the same significance as the "stormy fermentation" of *Clostridium perfringens*, the "iron-gelatin" reaction of *Clostridium histolyticum*, and the "vanillin-violet" (skatol) reaction of *Clostridium sporogenes* and *Clostridium parabotulinum* (Spray, 1936).

The conditions under which granulose is formed have been a matter of considerable study. We mention here only the study of Svartz (1930), who states, "The typical iodophil, clostridium-forming bacteria in the feces never deposited iodophil substance in a medium with a pH of less than about 6.6, even when there was plenty of carbohydrate in the medium." This held true also for a series of butyrics from various sources.

We have confirmed this statement in regard to a variety of butyrics obtained through the courtesy of Dr. Elizabeth McCoy and Dr. Ivan Hall—including McCoy's *Clostridium saccharobutyricum* (Wis. 63), *Clostridium felsineum* (Wis. 41), *Clostridium pasteurianum* (Wis. 60), *Granulobacter saccharobutyricus-immobile-non-liquefaciens* (Wis. 24), *Clostridium butyricum-iodophilum* (Wis. 61), *Clostridium butylicus* (Wis. 39), "unknown butyric" (Wis. 33), *Clostridium acetobutylicum* (Wis.), *Clostridium roseum* (Wis. 43), and a series of "unknown butyrics" from Dr. Hall, nos. 9039, 9041, 3234, 7241, 3813, 274, 9040, 9042, and 9043. However, these facts did not seem to apply to certain strains, including

[1] Professor Emeritus. Present address Box 742, Cumberland, Wisconsin.

C. pasteurianum (Wis. 5), *Clostridium beijerinckii* (Wis. 68), *C. beijerinckii* (ATCC 858), and Hall's "unknown butyrics" nos. 1067, 1334, 3815, and 3235.

TABLE 1

Estimation of growth determined by turbidity and gas formation at 17 hours' incubation

CULTURE	TUBE NO.													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	GLUCOSE %													
	2.0	1.8	1.6	1.4	1.2	1.0	0.8	0.6	0.4	0.2	0.1	0.05	0.025	0.00
Wis. 5	4	4	4	4	4	4	4	3	2	1	1	±	?	–
Wis. 60	4	4	4	4	4	4	4	3	2	1	1	±	?	?
Wis. 24	4	4	4–	3	2	2	2	2	2	1	1	±	±	?
Wis. 39	4	4	4	4	4	4	4	3	2	1	1	±	?	?
Wis. 68	4	4	4	4	4	4	4	4	3	2	1	±	?	?
Wis. 61	4	4	4	4	4	4	4	3	2	1	1	1	±	?

TABLE 2

Granulose reaction at 17 and 41 hours' incubation by the spot plate iodine test

CULTURE		TUBE NO.													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		GLUCOSE %													
		2.0	1.8	1.6	1.4	1.2	1.0	0.8	0.6	0.4	0.2	0.1	0.05	0.025	00.0
No. 5	17 hr	4	4	4	4	4	4	4	3	2	1	±	?	–	–
	41 hr	±	3	4	3	2	3	2	1	1	–	–	–	–	–
No. 60	17 hr	–	–	–	?	?	–	–	–	–	–	–	–	–	–
	41 hr	–	–	–	–	–	–	–	–	–	–	–	–	–	–
No. 24	17 hr	–	–	?	?	?	?	?	?	–	–	–	–	–	–
	41 hr	–	–	–	–	–	–	–	–	–	–	–	–	–	–
No. 39	17 hr	–	2	?	2	3	±	±	?	–	–	–	–	–	–
	41 hr	–	?	?	–	–	–	–	–	–	–	–	–	–	–
No. 68	17 hr	4	4	4	4	4	4	4	4	3	2	1	±	–	–
	41 hr	3	2	3	2	3	2	2	1	±	–	–	–	–	–
No. 61	17 hr	–	–	?	?	?	–	?	–	–	–	–	–	–	–
	41 hr	–	–	–	–	–	–	–	–	–	–	–	–	–	–

We proceeded then to a more intimate study of the phenomenon, applying the iodine test to semisolid glucose agar cultures. A sugar-free base was prepared (1 per cent Difco neopeptone, 1 per cent Difco tryptone, and 0.25 per cent agar in tap water). To one-half of this was added 2 per cent glucose, and dilu-

tions were made with the sugar-free portion, giving glucose concentrations of 2.0, 1.8, 1.6, 1.4, 1.2, 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, and 0.025 per cent, and a sugar-free control. The final reaction after autoclaving in tubes was about pH 7.2.

These glucose dilutions were inoculated with selected strains from the group enumerated above, and all tubes were incubated at 37 C. After 17 hours' incubation all tubes were examined for presence and amount of growth and gas, as recorded in table 1. At the same time samples were aseptically removed with capillary pipettes and tested for granulose on a spot plate, 2 drops of Gram's iodine being added to 5 drops of culture and stirred. The darkest violet reaction

TABLE 3

Beckman pH determinations and granulose reactions at 117 hours' incubation; iodine added to shaken tube cultures

CULTURE		TUBE NO.													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		GLUCOSE %													
		2.0	1.8	1.6	1.4	1.2	1.0	0.8	0.6	0.4	0.2	0.1	0.05	0.025	0.00
No. 5	pH	4.38	4.17	4.15	4.26	4.30	4.33	4.41	4.55	4.78	5.17	5.70	6.04	6.30	6.69
	Grn.	4	4	4	4	4	4	4	3	2	±	−	−	−	−
No. 60	pH	5.08	5.05	5.04	5.06	5.09	4.82	4.73	4.78	4.92	5.32	5.79	6.24	6.45	6.72
	Grn.	−	−	−	−	−	−	−	−	−	−	−	−	−	−
No. 24	pH	5.06	5.04	5.10	5.01	4.95	4.86	4.75	4.80	4.94	5.34	5.88	6.23	6.45	6.69
	Grn.	−	−	−	−	−	−	−	−	−	−	−	−	−	−
No. 39	pH	5.10	5.14	5.04	5.18	5.20	4.78	4.78	4.80	4.87	5.43	5.92	6.27	6.46	6.76
	Grn.	−	−	−	−	−	−	−	−	−	−	−	−	−	−
No. 68	pH	4.61	4.64	4.68	4.69	4.64	4.66	4.71	4.89	5.00	5.46	6.02	6.48	6.55	6.74
	Grn.	4	4	4	3	4	3	3	2	±	?	−	−	−	−
No. 61	pH	5.07	5.05	5.11	5.02	4.97	4.90	4.81	4.83	4.93	5.36	5.86	6.24	6.46	6.68
	Grn.	−	−	−	−	−	−	−	−	−	−	−	−	−	−

was recorded as 4 (++++), and less color in terms of comparative intensity as 3, 2, 1, ±, and −. The spot plate test was repeated after 41 hours' incubation, and the results of the two series are presented in table 2.

Finally, after 117 hours' incubation the pH of each tube was determined by the Beckman potentiometer, after which the entire contents of each tube were tested for granulose by shaking up the culture and then adding from 5 to 25 drops of Gram's iodine to each tube. In this test an initial strong reaction was followed by rapid fading. Further additions of 5 drops delayed this fading, until with 25 drops (to about 7 ml of medium) a relatively stable color was developed.

An attempt was made to measure this color in rather definite terms of starch-

iodine color. A 1.0 per cent solution of soluble starch (Difco) was accurately prepared in distilled water. From this were prepared 10 dilutions and a starch-free control. To tubes of these dilutions, in the same volume as the cultures, the same amounts of iodine were added and the colors recorded in similar terms. These starch-iodine tubes, as color standards, were used for comparison with the culture reactions. Incidentally, we found that the soluble starch gave a color

TABLE 4

Soluble-starch-iodine reactions (standards)

TUBE NO.	1	2	3	4	5	6	7	8	9	10	11
Soluble starch %	1.00	0.50	0.25	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.000
Starch-iodine reaction	4	4	4	4	4−	3	2	2−	1	?	−

TABLE 5

Granulose reactions at 117 hours' incubation recorded in terms of equivalent soluble-starch-iodine reactions (table 4)

CULTURE	TUBE NO.													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Wis. 5	0.5	0.5	0.50	0.25	0.25	0.25	0.125	0.063	0.016	0.002	0.00	0.00	0.00	0.00
Wis. 68	0.5	0.5	0.125	0.063	0.063	0.063	0.032	0.032	0.008	0.002	0.00	0.00	0.00	0.00
Wis. 60 Wis. 24 Wis. 39 Wis. 61	0.0	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

TABLE 6

Significant fermentations of 1 per cent carbohydrates in semisolid agar base

CULTURE	LACTOSE	CORN-STARCH	DEXTRIN	GLYCOGEN	SALICIN	GLYCEROL	MANNITOL	SORBITOL	DULCITOL	INOSITOL
Wis. 5	−	−	−	−	−	+	+	+	−	+
Wis. 60	+	+	+	+	+	−	+	+	+	+
Wis. 68	+	−	−	−	+	−	+	+	−	+

reaction almost exactly comparable to that of the granulose. Similar dilutions of cornstarch gave a distinctly bluer color, much less satisfactory for comparison.

The results of these tests are recorded in tables 3, 4, and 5, only for the selected significant cultures: *C. pasteurianum* (Wis. 5), *C. pasteurianum* (Wis. 60), *Granulobacter saccharobutyricus–immobile–non-liquefaciens* (Wis. 24)—incidentally, a misnomer for this strain, both because of the name and the fact that the

culture is actually motile, *C. butylicus* (Wis. 39), *C. beijerinckii* (Wis. 68), and *C. butyricum-iodophilum* (Wis. 61).

Of the other cultures enumerated above, and not included in the tables, the Wisconsin series 63, 41, 33, *C. acetobutylicum*, and 43, and Hall's "unknowns" 9039, 9041, 3234, 7241, 3813, 274, 9040, 9042, and 9043 fall in the granulose-negative group. The ATCC 858 and Hall's "unknowns" 1067, 1334, 3815, and 3235 fall in the granulose-positive group.

From these reactions of selected, representative species there appear three interesting observations: (1) the granulose reaction may be determined conveniently and sharply in a semisolid glucose agar, (2) this reaction is not necessarily inhibited by a strong acidity (as low as pH 4.15), and (3) the test by the method given seems to distinguish two species (*C. pasteurianum*, Wis. 5, and *C. beijerinckii*, Wis. 68) from all of the other butyric anaerobes tested.

It should be noted that *C. pasteurianum* (Wis. 60) does not show the granulose reaction. The explanation appears in the results of the fermentation tests with the various carbohydrates (table 6). Only the significant differential carbohydrates are included.

My reactions of Wis. 5, originally from Winogradsky, check with those of McCoy *et al.* (1930), but deviate from Winogradsky's (1902) original description of *C. pasteurianum* regarding glycerol, mannitol, and dextrin. Such divergences may be due to methods or to strain variations, of which he observed several.

Strain Wis. 60, a Lister Institute strain from Bredemann, is obviously not a true *C. pasteurianum* type, especially as shown by the fermentation of both lactose and cornstarch. It appears, therefore, to be merely one of the many ill-defined butyrics, probably close to the "*B. saccharobutyricus*" type of McCoy *et al.* (1926). Strain Wis. 68, originally from Kluyver, checks with the limited original description of *C. beijerinckii*, named by Donker (1926). This strain is obviously distinct from both Wis. 5 and Wis. 60.

SUMMARY

A method of testing for granulose in a semisolid glucose agar is described.

This reaction appears to set two butyrics (types or "species"), *Clostridium beijerinckii* and *Clostridium pasteurianum*, apart from all other members of the group included in this study.

This reaction seems to have a differential value equivalent to the "stormy fermentation" of *Clostridium perfringens*, the "iron-gelatin" reaction of *Clostridium histolyticum*, and the "vanillin-violet" (skatol) reaction of *Clostridium sporogenes*.

For the two species giving the positive granulose reaction the pH 6.6 limitation of Svartz does not apply. It does, however, appear to do so for all other butyrics included in this study.

These two strains formed granulose, in diminishing amounts, detectable in all glucose dilutions from 2.0 per cent through 0.05 per cent, inclusive.

That these reactions are fixed, and not evanescent, characters is proved by the fact that the same strains, separated in the laboratories of Dr. McCoy, Dr. Hall,

and the author for 7, 9, and even 11 years, have proved identical in every particular in duplicate and in triplicate cultures.

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