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SOME FACTORS AFFECTING THE TOXICITY OF CULTURES OF SHIGELLA DYSENTERIAE

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Cultures of *Shigella dysenteriae* are extremely toxic to man and experimental animals; it is likely that this toxicity is of great significance in determining the symptomatology and pathology of Shiga dysentery. Many conflicting views have been voiced to account for it. Some authors, for instance, feel that all the toxic manifestations are due to one single substance.[1] Others, on the contrary, believe in the existence of two independent toxins [2-8]: (*a*) an endotoxin, which exhibits a special affinity for the intestinal tract, is present only in the smooth variants of the organism, is associated chemically with the specific somatic polysaccharide and is resistant to heat and to proteolytic enzymes; (*b*) an exotoxin with neurotropic affinity, which is present both in the rough and smooth variants, is of protein nature and is inactivated by heat and trypsin. It has also been claimed that aerobic incubation favors the production of the exo-neurotoxin whereas only the endotoxin is produced under anaerobic conditions.[2]

It is obvious that, before any final claim can be made concerning the nature and pathological activity of the toxic components of the Shiga bacillus, methods have to be devised for the production in large quantities and for the purification of these substances. In view of the practical importance of this problem for the eventual development of immunization procedures, we are reporting at the present time some of our preliminary observations which may serve to define better the optimal conditions for the production of toxin and the properties of at least one of the toxic fractions.

Effect of Cultural Conditions on the Toxicity of Cultures of Shigella dysenteriae.—All experiments were performed with strain 32158 of *Shigella dysenteriae* which was obtained through the courtesy of Miss M. Coleman of the New York State Department of Health. Two phase variants were used: (1) the smooth form (*s*) which gave typical agglutination in standard antisera, and (2) a rough variant (R_{12}) which was isolated from an old stock

culture. The organisms grown in liquid media were collected by centrifugation or filtration (in the case of the *R* variant); the agar cultures were suspended in distilled water and also centrifuged. It was observed that in the case of young cultures at least (24 to 48 hours old, for instance), only a very small percentage of the total toxicity of the whole culture was lost in the culture supernatant or cell washings; in other words, the toxic components of young cultures seemed to be bound to the cell structure, a fact already observed by others.[9]

The dry weights of the cultures were obtained by desiccation over phosphorus pentoxide *in vacuo*, or by treatment with acetone ether; it was found that these methods of desiccation did not alter the toxicity of the original material.

A number of different toxicity tests were used: minimal lethal dose for rabbits and for white mice (20 grams), production of the Shwartzman reaction in rabbits,[10] production of the ocular reaction in rabbits,[11] etc. Rabbits were found to be much more susceptible than mice; for reasons of economy, however, most titrations of toxicity were based on determination of the minimum lethal dose for mice, and only the results of these tests will be reported at the present time.

The effect of cultural conditions on toxicity is well illustrated by the following experiment. Cultures of rough and smooth variants of Shiga bacillus were grown at 37° for 18 hours in meat infusion peptone broth, (the layer of fluid being 10 cm. thick), or on meat infusion peptone agar. The washed cells were desiccated *in vacuo* over phosphorus pentoxide, resuspended in 0.05 *M* phosphate buffer at pH 7.2, heated at 60°C. for 10 minutes, and injected intraperitoneally into white mice. The results presented in table 1-A indicate that in the case of both the rough and smooth variants, 0.15 mg. was the minimal lethal dose when the agar cultures were used, and 1.5–2.0 mg. with the broth cultures.

Aqueous extracts of agar were added to meat infusion peptone broth to determine whether there is present in agar a water-soluble component which favors toxin production; no increase in toxicity of the broth culture, however, resulted from this treatment.

It appeared possible that the greater production of toxin on agar media was due to the fact that the agar gel permits surface growth and therefore increases aerobic metabolism. To test this hypothesis, media were prepared in which the solid surface was supplied by silica gel[12] instead of agar. To the silica gel were added 0.05 *M* phosphate buffer at pH 7.4, 1.0 per cent peptone and 1.0 per cent meat extract. The medium was poured into petri dishes, some of the plates receiving 1.0 per cent dextrose in addition to the other components of the medium. The plates were inoculated with rough or smooth variants of Shiga cultures; after 18 hours' incubation, the growth (which was less abundant than on agar) was collected from the

TABLE 1

TOXICITY OF DESICCATED CELLS OF *R* AND *S Shigella dysenteriae* GROWN UNDER DIFFERENT CONDITIONS

CULTURE	MEDIUM	NUMBER OF MICE SURVIVING I.P. INJECTION OF FOLLOWING AMOUNTS OF CELLS (MG. DRY WEIGHT)			
	1-A				
		1.5	1.0	0.3	0.1
R	Meat infusion peptone agar	0/3[a]	0/3	0/3	1/3
R	Meat infusion peptone broth	2/3	2/3	3/3	3/3
S	Meat infusion peptone agar	0/3	0/3	0/3	0/3
S	Meat infusion peptone broth	2/3	3/3	3/3	3/3
	1-B				
		1.0	0.5	0.25	0.1
R	Nutrient silica gel	0/1	0/2	0/3	0/2
R	Nutrient silica gel + 1% glucose	0/1	0/2	2/3	3/3
S	Nutrient silica gel	...	0/3	0/3	0/3
S	Nutrient silica gel + 1% glucose	...	0/3	2/3	3/3
	1-C				
		1.5	0.75	0.45	0.15
R	Peptone broth mechanically shaken (total yield 600 mg./liter)	0/3	0/3	1/3	2/3
R	Anaerobic peptone broth (total yield 200 mg./liter)	1/3	3/3	3/3	3/3
	1-D				
		1.5	0.75	0.30	0.15
R	Peptone broth mechanically shaken (total yield 750 mg./liter)	...	0/3	2/3	3/3
R	Thioglycollate peptone broth (total yield 420 mg./liter)	3/3	3/3	..	..

[a] The denominator indicates the number of mice used in the test. The numerator indicates the number of mice surviving after 10 days.

silica gel surface, washed with water, desiccated with acetone ether, resuspended in neutral phosphate buffer, heated at 60°C. for 10 minutes and injected intraperitoneally into white mice. The results presented in table 1-B indicate that, in the case of both the rough and smooth variants, the cells grown on silica gel medium not containing glucose were extremely toxic, even more toxic than the cultures grown on agar media. Addition of glucose to the medium markedly reduced toxicity.

Since surface growth gives rise to cells much more toxic than the cells grown in liquid media, an attempt was made to increase oxidative conditions within the broth by bubbling oxygen during the whole course of incubation; these conditions brought about some increase in toxicity, but the

results were not striking enough to warrant further description at the present time.

In other experiments, the inoculated medium was violently agitated on a shaking machine during the whole period of growth. It was found that, not only did the shaking markedly increase the total yield of cells, but that the toxicity per milligram of dry weight of cell was also greatly increased. These facts are illustrated in the two following experiments.

A medium containing 0.05 *M* phosphate at pH 6.9, 1.0 per cent peptone (Difco tryptone) and 1.0 per cent glucose was inoculated with a rough variant of *Shigella dysenteriae*. Half of the medium was incubated in an aerobic jar;[13] the other half was violently agitated on a shaking machine during the whole incubation period. The cells were collected by centrifugation after acidification at pH 4.5 (a reaction at which none of the broth constituents are precipitated), washed with water and desiccated with acetone ether. The total yields were approximately 600 mg. per liter in the case of the aerobic culture, and only 200 mg. in the case of the anaerobic culture. The cells were resuspended in neutral phosphate buffer, heated at 60°C. for 15 minutes and injected intraperitoneally into white mice for a determination of comparative toxicity (table 1-C).

In another experiment anaerobic conditions of incubation were obtained by the addition of 0.05 per cent of sodium thioglycollate to the medium, and aerobic conditions sccured by constant agitation. The medium consisted of 1.0 per cent tryptone (Difco), 1.0 per cent glucose, 0.05 *M* phosphate at pH 7.8, and was inoculated with a rough variant of Shiga bacillus (table 1-D).

The results presented in tables 1-C and 1-D indicate clearly that anaerobic incubation gives yields of cells very much smaller than those obtained under conditions of aerobic agitation during growth (750 mg. per liter versus 420). The toxicity of the cells grown under aerobic conditions is also much greater per milligram dry weight than that of cells obtained by anaerobic incubation.

Separation of a Toxic Component from Cultures of a Rough Variant of Shigella dysenteriae.—Large amounts of culture of the variant form R_{12} of the *Shigella dysenteriae* were grown on meat infusion peptone agar at 37°C. for 18 hours; the cells were washed in water and desiccated *in vacuo* over phosphorus pentoxide. The average yield was approximately 20 mg. of washed dried cells per agar plate (8 cm. diameter); 0.15 mg. of dried cells (heated at 60°C. for 15 minutes in 0.05 *M* neutral phosphate buffer) was the minimal lethal dose for mice. The bacterial component responsible for the toxicity of the culture exhibits the following properties: (1) it is only slowly inactivated by heat at neutral and at acid reactions, but is very rapidly destroyed at alkaline pH; (2) it is completely resistant to proteolytic enzymes (crude trypsin, pepsin, papain, mold and bacterial en-

zymes); (3) it is precipitated by two-thirds saturation with ammonium sulfate, and by 33 to 50 per cent acetone; (4) it is precipitated at acid pH (pH 3.0 to 4.0); (5) it can be obtained in solution in 0.05 *M* dibasic phosphate from heat-killed cultures digested with trypsin; (6) the addition of papain to a solution of the toxic material causes the precipitation of an inactive fraction (probably nucleic acid), whereas the active material remains in solution; it can be precipitated again from the papain solution by addition of acid; (7) the active principle does not dialyze through cellophane membranes.

By making use of these different properties, we have obtained fractions of such activity that 3.0 micrograms injected intraperitoneally into white mice, or intravenously into rabbits, regularly causes death within 3 to 4 days; death of mice was also observed with amounts as small as 1.3 microgram. The material is stable and retains its activity unaltered for several weeks when kept in neutral solution at 5°C.

Conclusions.—It has been shown that conditions which favor aerobic metabolism (growth on agar or on silica gels, or in broth violently agitated during incubation) greatly increase the yield of *S. dysenteriae* cultures and the toxicity of the cells (measured in terms of dry weight). For instance, 1.5 mg. of dried cells grown in an anaerobic jar failed to kill any mice, whereas 0.1 mg. of cells grown on silica gel plates killed all mice (smaller amounts were not tried). The addition of glucose to silica gel medium caused a definite decrease in the toxicity of the cells; this may be due to the fact that, even when growth takes place under aerobic conditions, the presence of glucose permits some anaerobic metabolism.

The effect of environmental conditions of growth on the yield of organisms and on the yield of toxin was the same for rough and for smooth variants of *Shigella dysenteriae.* In all cases also, the largest percentage of total toxicity of the whole culture was found to be bound to the bacteria bodies and to be released in solution only as a result of autolysis.

The toxic factor is resistant to proteolytic enzymes; it can be obtained in the form of a water-soluble fraction which is stable and of which 1.0 to 3.0 micrograms cause the death of mice and rabbits within 72 hours.

* Preliminary experiments were carried out in the laboratories of the Hospital of the Rockefeller Institute, New York.

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THE FUSION OF BROKEN ENDS OF CHROMOSOMES FOLLOWING NUCLEAR FUSION

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When, through radiation or other causes, chromosomes are broken within a single nucleus, 2-by-2 fusions may occur between the broken ends. These fusions may lead to rearrangements of parts of the chromatin complement, giving rise to various chromosomal aberrations which are detected as reciprocal translocations, inversions, deficiencies, etc. Since, in the well-investigated cases, the breakages occurred within a single nucleus, the conditions that lead to fusions of broken ends could not easily be ascertained. The following questions have been asked: (1) Must two or more chromosomes be in intimate contact at the time of breakage in order that fusions may occur? (2) If no intimate contact is necessary at the time of breakage, are the broken ends "unsaturated," that is, capable of fusion with any other unsaturated broken end? (3) If question (2) can be answered in the affirmative, what forces are involved which lead to the contact and subsequent fusions of the two unsaturated broken ends? Likewise, (4) how long will these broken ends remain unsaturated, i.e., capable of fusion?

Questions (1) and (2) could be answered if the following conditions were present: Assume that fusion occurs between two nuclei each of which possesses one chromosome, one end of which has been broken. Each nucleus will then have a single broken end. When these nuclei fuse and their chromosomes intermix within a single nucleus, the chromosome with a broken end contributed by one nucleus could fuse with the chromosome with a broken end contributed by the second nucleus. The chromosome fusion should occur between these two broken ends. This experiment may easily be conducted in maize. The two nuclei that fuse can be the male and the female gametes, respectively. The method of obtaining