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THE PREPARATION OF A POTENT TOXIN OF SHIGELLA DYSENTERIAE (SHIGA) ON A SEMI SYNTHETIC MEDIUM AND ITS USE FOR THE PREPARATION OF AN ALUM-PRECIPITATED TOXOID

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The first attempt to immunize against the toxin of Shigella dysenteriae by anatoxin was reported by Dumas, Ramon and Bilal (1). They used a filtrate from a 15 day broth culture grown at 37 C. Of this filtrate, 0.1 ml killed a rabbit in 2–3 days, but after a 40 days incubation with 0.4 per cent formaldehyde at 37 C, 10 ml of the filtrate caused no harm when injected intravenously and immunized the animal against several lethal doses. In subsequent experiments (2) toxins with minimal lethal doses of 0.04–0.025 ml were used. 0.24 per cent formaldehyde was added to the toxin and the incubation time was reduced to 30 days. Two injections of 0.5 and 1.0 ml of the anatoxin over an eight day interval immunized against 6–8 minimal lethal doses of toxin given intravenously.

Halapine and Basilevskaia (3) prepared potent toxins in 2–3 liter flasks containing 600–900 ml broth. After a 15 days incubation at 37 C, the minimal lethal dose was 0.01 ml for rabbits and 0.2–0.02 ml for mice. After a 60 days incubation with 0.2 per cent formaldehyde at 39–40 C, 10 ml of the anatoxin was toxic for rabbits and 0.5 ml given intravenously killed 50 per cent of mice. Anatoxin prepared by three month incubation with 0.24 per cent formaldehyde was also not completely detoxified. On the other hand, filtrates from 48 broth cultures were easily detoxified; after 15 days incubation the minimal lethal dose of such filtrates was reduced from 0.01 ml to 0.1 ml. Anatoxins were employed for immunization of horses and antitoxic sera containing 1500–10000 A.U. were obtained. O'Brien (4) recommends the use of formolized Shiga-toxin for the production of immune serum in horses, because untreated toxin may cause severe local swelling and a general reaction in horses. He confirmed that the formol toxoid is a good antigen and that it is less irritant than the untreated toxin. L. Farrell (5) used autolysates of bacteria grown on agar instead of filtrates from broth cultures. These toxins had a minimal lethal dose of 0.001–0.002 ml for mice. They were detoxified by formaldehyde "under suitable conditions." Rabbits received three injections of 6 ml and developed 10–20 units of antitoxin per ml of serum. Nine human subjects, 7 of them convalescents, received 0.5 ml of the same preparation. Three of the subjects developed slight headache and one of them chills and dizziness. 0.75 ml evoked in normal persons a production of 1.5–2 units antitoxin per ml.

Recently Farrell, Fraser and Ferguson (6) reported a trial of dysentery toxoid (Shiga) in human volunteers. Three doses of toxoid (0.25–0.5 ml) were given.

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For practical purposes, a 3-week interval between injections and a recall dose three months later were proposed. Ten days after the third injection 88–94 per cent of the persons had an antitoxin titer of 0.2 units per ml. Reactions other than headache and sore arm were not observed. Olitzki and Koch (7) succeeded in producing a potent dysentery toxin in a medium consisting of pure amino acids, salts and other known chemicals. In further experiments (8), they prepared an anatoxin from the filtrate of a semisynthetic medium. Three injections of 0.1 ml of this toxoid protected mice against intraabdominal infection with 2×10^6 bacteria (suspended in 5 per cent mucin). During an epidemic in September 1942, 1400 persons, 900 of 12–18 and 500 of 8–12 years, who had been heavily exposed to Shiga-infection received three injections of 0.5, 1.0 and 1.0 ml of toxoid at 8 day intervals. On the days following the injections temperatures from 37.2–37.6 were observed. The main symptoms observed following treatment were headache, local aches at the place of the injection and in 1–2 per cent of the subjects a transitory weak diarrhoea. In the 6 months following vaccination no cases of dysentery were reported in the treated group. The work of Farrell *et al* (5, 6) and the immunization experiment carried out by Olitzki and Koch (8) in 1400 persons show that dysenteria toxoid does not produce any serious symptoms in human beings, and that it can be useful during an outbreak even in a highly exposed population.

The purpose of the work reported here was to examine the effect of different conditions, e.g. aeration and time of incubation upon the production of a potent toxin in a medium suitable for large scale work. Such a medium should answer the following requirements: it must be inexpensive, readily prepared, capable of yielding a potent toxin and easily reproducible. The toxin must be tested for antigenicity, detoxification by formaldehyde, and immunizing power after detoxication and adsorption on alum.

In the experiments described below *S. dysenteriae*, "Bukarest S" was employed.

The preparation of the medium. The medium was prepared from four stock solutions: an acid casein hydrolysate, an alkaline casein hydrolysate, a salt solution and glucose solution.

1. *Acid casein hydrolysate.* To 50 g of casein, 250 ml of 25 per cent sulfuric acid were added. The mixture was autoclaved at 15 lbs for 10 hrs, neutralized with $Ba(OH)_2$ and brought to a total volume of 450 ml. This solution contained 13.25 mg N in 1 ml.

2. *Alkaline casein hydrolysate.* To 50 g casein, 500 ml of a 10 per cent $Ba(OH)_2$ solution were added. The mixture was autoclaved at 15 lbs for 10 hrs and neutralized with sulfuric acid. The hydrolysis by alkali was not complete. The final product contained 6.10 mg N in 1 ml.

3. *Salt solution.* This solution was autoclaved at 15 lbs for 30 minutes. It contained (expressed in percentages) KH_2PO_4 0.5, Na_2HPO_4 4.5, $NaNO_3$ 0.5.

4. *Glucose solution.* A 10 per cent glucose solution was autoclaved at 10 lbs, for 15 minutes and kept in the ice box.

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Final medium. The final medium contained 50 ml of solution 1 50 ml of solution 2, and 100 ml of solution 3. After the pH was adjusted to 7.2–7.3, the total volume was brought to 1L and the medium autoclaved at 15 lbs for 30 min. 10 ml of solution 4 were added per liter of the medium under sterile conditions immediately before the inoculation of the bacteria. Thus the final medium contained: casein hydrolyzed by acid corresponding to 0.5 per cent, casein hydrolyzed by alkali corresponding to 0.5, KH_2PO_4 0.05 per cent, Na_2HPO_4 0.45 per cent, $NaNO_3$ 0.05 per cent, glucose 0.1 per cent.

Inoculum. 0.2 ml of a 24 broth culture of *S. dysenteriae.*

The importance of individual constituents of the medium for bacterial growth and toxin production. The following tests were made in media prepared without additions of certain of the constituents mentioned above. The maximal yield on whole medium after maximal aeration continued 48 hrs at 30 C was 540 mg bacterial substance per liter of medium. The importance of the various constituents is demonstrated by the following figures:

MEDIUM	YIELD OF BACTERIAL SUBSTANCE PER LITER	PER CENT GROWTH	pH
	mg		
Original medium	540	100.0	7.4
Medium without glucose	340	64.8	8.0
Medium without casein hydrolyzed by acid	370	68.5	6.6
Medium without casein hydrolyzed by alkali	380	70.4	7.6
Medium without $NaNO_3$	290	53.5	8.0
Medium without KH_2PO_4	520	98.1	8.3
Medium without Na_2HPO_4	180	33.3	5.6

The question arises whether toxin production is inhibited in the presence of glucose because this sugar induces excessive acid formation or because the presence of glucose brings about a modification of the metabolism of *S. dysenteriae.* According to Dubos *et al* (9) the diminution of toxin production in the presence of glucose is due to anaerobic metabolic processes which are unfavorable to toxin production. The following experiment confirms this view: the bacteria were grown on the semi-synthetic medium described above in the presence of 1.0 per cent glucose with optimal aeration. After 24 hours the reaction was brought to pH 7.5. No further acidification occurred. After an 8 day incubation at 30 C 730 mg bacterial substance was obtained per liter, but the minimal lethal dose of the toxin was 0.2 ml. If we compare this figure with those obtained with a medium containing 0.1 per cent glucose, namely 540 mg bacterial substance in 1L with a minimal lethal dose of 0.05 ml, it becomes evident that toxin formation does not parallel bacterial growth and that toxin production is markedly reduced in the presence of 1 per cent glucose even if the acid is neutralized. This result is confirmed by the fact that a toxic filtrate

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kept at 37 C at pH 6.0 for 24 hours did not show any reduction of toxicity; the toxin was damaged only at a pH below 5.0. It seems therefore that acid production from glucose is not the main factor which prevents toxin production.

Influence of aeration. Maximal aeration is a fundamental condition for the production of dysentery toxin. The following experiments show the influence of aeration on bacterial growth within 48 hours at 30 C. Different quantities of the semi-synthetic medium were divided into Erlenmeyer flasks of 1 liter content. One part of the cultures was aerated by a steady air current during 48 hours, the other cultures were allowed to grow without aeration. The following results were observed:

	MEDIUM	BACTERIAL SUBSTANCE IN 1L	pH
	ml	*mg*	
Aerated cultures:	500	250	7.2
	200	380	7.4
	100	540	7.6
Not aerated cultures:	500	80	6.8
	200	110	7.0
	100	120	7.4

The experiments show that the best growth is obtained in Erlenmeyer flasks of 1 liter each containing only 100 ml of culture medium aerated by an air current. Maximal aeration is important in preventing anaerobic metabolism and acid presence unfavorable to toxin production. The minimal lethal doses of toxins produced from 100 ml aerated cultures after 8 day incubation at 30 C were from 0.05 ml to 0.02 ml when given intravenously to rabbits of 1000 g weight; with non-aerated cultures the minimal lethal doses were never less than 0.5 ml to 0.2 ml.

The maintenance of a pH of 7.4 represents an optimal point according to numerous experiments of different authors and our own experiments. According to Olitzki and Koch (7) even 0.1 per cent glucose reduces toxin production in a synthetic medium poor in N-containing substances. In the medium described above the action of the glucose is evident only if acid casein hydrolysate or secondary phosphate are omitted; in this case the pH decreases to 6.6 and 5.6 respectively and a poor bacterial growth with low toxicity results. The primary phosphate had only a weak effect on the growth, but its presence prevents excessive alkali formation.

Incubation period and detoxification. After a 48 hrs incubation at 30 C with maximal aeration as described above, no further increase of bacterial substance was observed. The cultures were allowed to remain at the same temperature for a further 6 days without aeration. According to earlier experiments of Olitzki *et al* (10), this should be long enough to permit a sufficient degree of autolysis and release of toxin into the culture fluid. According to earlier

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experiments, longer incubation favours the release of increased amounts of "enterotoxin," a factor which is not detoxicated by formalin according to Lubinski and Pfeiffer (11). The negative experiments of Halapine and Basilevskaia (3) with old cultures and the positive results obtained with young cultures may be explained by the presence of the enterotoxin in the former and its absence in the latter.

After an incubation period of 8 days at 30 C the cells were removed by centrifugation and sufficient formaldehyde was added to the supernate fluid to produce a concentration of 0.08 per cent. After the fluid has been stored at 30 C for 21 days, detoxification was complete. 5 ml injected intravenously failed to kill rabbits weighing 1 kg.

Toxin after dialysis has not lost its toxicity and contains 0.56 mg N per ml. while the original medium after dialysis contained 0.11 mg N. It may be concluded, therefore, that at least 0.45 mg of non-dialysable N were formed by the bacteria.

Adsorption on alum. To equal portions of toxoid or toxin (1 ml) various quantities of a 10 per cent alum-suspension were added and the mixtures left overnight in the ice box. The following quantities of N were found to be adsorbed on the alum (which was removed by centrifugation):

Final concentration of alum in per cent:	5.0	3.0	2.0	1.0	0.5	0.2	0.1
mg N absorbed from 1 ml.	0.07	0.16	0.15	0.33	0.09	0	0

A maximal quantity of non-dialysable N (0.33 out of 0.45 mg) was adsorbed by 1 per cent alum. Toxicity tests on the supernatant fluid (after treatment with 1 per cent alum) showed that the minimal lethal dose increased to 0.1 ml from 0.05 ml for the original toxin. The observation suggests that the adsorption of toxin parallels that of non-dialysable N.

For immunization tests, 1 per cent of alum was added to toxoid. The suspension was shaken thoroughly and the mixture allowed to stay overnight in the ice box. The precipitate was removed and washed by centrifugation twice with saline, and once more with 0.5 per cent Na_2HPO_4, and a third time in saline. The sediment was resuspended in the required quantity of saline.

Immunization experiments. The experiments showed that the immunizing effect is determined mainly by the potency of the toxin from which the anatoxin is derived. A weak toxin with a minimal lethal dose of 0.5 ml for rabbits was prepared from a non-aerated culture. The anatoxin prepared from this toxin had the following immunizing effect: Three subcutaneous injections of 2.0, 4.0 and 4.0 ml protected rabbits against 1 minimal lethal dose; three injections of 4.0, 8.0 and 8.0 ml respectively immunized against 4 MLD of toxin. Adsorbed on 1 per cent alum and resuspended in saline in a quarter of its original volume this anatoxin produced the following effect: 0.05, 0.1 and 0.1 ml given subcutaneously protected 2 rabbits against 1 MLD and one rabbit against 2 MLD but it did not protect against 4 MLD. 0.1, 0.2 and 0.2 ml protected three rabbits against 2 MLD. 0.5, 1.0 and 1.0 ml protected 3 rabbits against 4 MLD but did not protect against 8 MLD.

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An anatoxin derived from a toxin with a minimal lethal dose of 0.05 ml for rabbits immunized rabbits against 8 MLD when 2.0, 4.0 and 4.0 ml of the anatoxin were injected. After adsorption on 1 per cent alum and concentration to $\frac{1}{4}$ volume, 0.5, 1.0 and 1.0 ml of the anatoxin immunized against 16 MLD.

Best results were obtained with an anatoxin derived from a well aerated culture. The minimal lethal dose of the original toxin was 0.02 ml for rabbits. As reported above, only 50 per cent–60 per cent of the anatoxin is adsorbed by 1 per cent alum. In order to obtain a 5 fold concentration, the anatoxin adsorbed by alum was removed by centrifugation and resuspended in a volume of saline corresponding to one tenth of the original volume. Rabbits received subcutaneous injections of 0.05, 0.1 and 0.1 ml of this concentrated anatoxin in 7 day intervals. 10 days after the last injection various quantities of toxin were given intravenously. The animals which had received 1.0, 2.5, 5.0 and 7.5 minimal lethal doses survived; those which had received 10 MLD succumbed. In another series, the animals received subcutaneous injections of equivalent amounts of the original toxoid (0.25, 0.5 and 0.5 ml). When injected with 1, 2.5 and 5 MLD of toxin all these animals succumbed. This test proves the superiority of the alum precipitated toxoid for immunization.

DISCUSSION

Our experiments show that it is possible to produce potent Shiga-toxin on a semi-synthetic medium and define the conditions which favor maximal toxin-production in such a medium. Aerobic conditions of growth are important; they greatly increase both the yield of cells and the toxicity. This important point was first emphasized by McCartney and Olitsky (12) who by suppression of the exotoxin-producing activity of *S. dysenteriae* by anaerobiosis produced pure endotoxin directly from the culture. Dubos, Hoberman and Pierce (9) demonstrated that conditions which favor aerobic metabolism (growth on agar or on silica gels, or in broth violently agitated during incubation) greatly increase the cell yield and toxicity of Shiga cultures. Olitzki and Koch (7) succeeded in producing potent Shiga-toxin on a synthetic medium in test tubes only if the test tubes were maintained during incubation in a nearly horizontal position. In the experiments reported above we obtained steady aeration during the 2 first days of incubation by the use of 1L Erlenmeyer flasks containing only 100 ml of medium and by the addition to the medium of 0.05 per cent of $NaNO_3$ which undergoes reduction to $NaNO_2$ and seems to favor aerobic metabolism.

According to some authors the reaction of the medium before and during incubation is a further factor influencing toxin production. Olitzky and Kligler (13) reported that in broth, that was not rendered sugar-free, exotoxin only appeared in considerable quantity when the period of initial acidification was followed by a period in which the reaction became alkaline (pH 7.4–7.6). Olitzki and Koch (7) also found that acid was produced in a synthetic medium at the beginning of the growth, but that the reaction became alkaline after 3–5 days. After an incubation of 10 days, optimum yield of toxin was observed. Addition of glucose enhanced the amount of acid produced and diminished the toxicity.

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The experiments reported above show that excessive acid may cause damage to the toxin, but they also show that acid production is not the main factor for the prevention of toxin production in the presence of large quantities of glucose. It seems that other more fundamental modifications of the bacterial metabolism influence the production of toxin.

Dubos *et al* (9) looked for an explanation for the effect of glucose in the fact that the presence of glucose permits some anaerobic metabolism, even if growth takes place under aerobic conditions. The experiments reported above prove that very small quantities of glucose (0.1%) are able to enhance bacterial growth and toxin production, if sufficient quantities of casein hydrolysate are present. When 1 per cent was employed the detoxifying effect described by Dubos *et al* (9) was observed. It seems therefore that the glucose concentration is a most important factor for toxin production. The concentration of glucose should not be above the level of providing the needed quantity of energy. There should also be present a sufficient quantity of alkali-producing substances for neutralization of the acid products of glucose fermentation. Furthermore, it is necessary to ensure a supply of oxygen sufficient to prevent anaerobic metabolic processes and to allow complete oxidation of the intermediary acid products in order that maximal utilization of the sources of energy may be realized.

Under optimal conditions a yield of 540 mg of bacterial substance per liter after an incubation of 2–3 days has been obtained. The production of a maximal quantity of toxic bacterial substance during the first 2 days of the incubation is essential for the appearance of toxin in the *filtrate*. Toxin liberation is not a function of the living cell, but is, according to Dubos *et al* (9) and also Olitzki *et al* (10), the result of autolysis. The immunization experiments confirm both the exotoxin-character of the Shiga-toxin, its detoxification by formalin, and its ability to immunize against a multiple lethal dose when injected in small quantities.

SUMMARY

Shiga toxoid was prepared from cultures of *S. dysenteriae* on a semi-synthetic medium. Small quantities of this toxoid adsorbed on alum, concentrated, and injected subcutaneously immunized rabbits against multiple lethal doses of toxin.

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