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On certain poisonous substances produced in bacterial cultures.

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In a paper published by one of us last year we called attention to the fact that it was quite likely that not all the toxic substances produced in cultures by bacteria can be peremptorily classified as either exotoxins or endotoxins. The writers working with a number of different organisms, biologically unrelated, have found poisonous substances of moderate potency developed both on fluid and on solid media which they believe should not be regarded at the present time either as specific or antigenic exotoxins, or as endotoxins. Indeed, it seems quite impossible at the present time to definitely classify these substances, for which reason they are referred to in our laboratory, for the present, as the "X" substances.

I.

Our first observations on this substance were made with Miss Kuttner on hemolytic streptococci. It was found that supernatent fluids or filtrates of such cultures grown for 20 to 22 hours either aërobically, or better, with partial anaërobiosis, regularly produced sickness in rabbits, although there was great irregularity in potency. Potency was never great. Rabbits intravenously injected with such culture fluids always showed an incubation period of from 60 to 90 minutes, at the end of which time a regular train of symptoms ensued, consisting of respiratory difficulty, weakness, refusal to eat, flattening out on the bottom of the cages, muscular relaxation, half closure and often watering of the eyes, in which condition they either remained for 2 to 3 hours, and then recovered, or in some cases died acutely in anywhere from 2 to 3 hours to two days. The dosage necessary for this ranged between 3 and 6 cubic centimeters. It was apparently impossible to control the potency of these substances. We have not found out the reason why, in some cases we obtained very severe symptoms and death, but in most instances only moderate, but distinct illness.

Experiment has shown that the poisons are not extractives of the bacterial bodies.

They are weakened and sometimes destroyed by heating to 80° for 30 minutes.

They seem to be relatively more potent for rabbits than for guinea pigs.

They cause a marked reduction of leucocytes and probably in this sense have a definite agressin-like action. Small doses of streptococci injected with such substances killed, in some instances, in a shorter time than did many times the amount of washed organisms of the same cultures.

In four separate experiments it has seemed distinctly as though the first isolation of certain streptococci from the human body gave rise to more potent poisons than we were ever able to produce with the same organisms in later generations.

Time experiments seem to show that the poisonous substance is more plentiful in the cultures at about the period of the highest growth energy of the organisms.

There is no indication that there is any relationship between these substances and virulence.

The substances were produced on simple hormone media with almost the same potency with which they appeared on the richer protein media such as chocolate broth. In general, however, the potency was in favor of the richer media.

Many of the more accurate determinations of biological properties, such as certainty about the temperature of destruction, deterioration at room temperature, and relative toxicity for various animals, are difficult to determine with absolute certainty because of the great fluctuation in potency of the substances obtained and the probable differences in susceptibility of individual rabbits.

In spite of fluctuations of potency, however, some degree of toxic action was never absent and its importance in connection with animal immunization necessitated further study.

As to the formation of these substances in the body of the animal, we can say, at the present time, only that the symptoms produced after an incubation of from 1 to 1½ hours in a rabbit are similar in general to those which appear in a rabbit at the time when the streptococci begin to appear in the blood stream in considerable numbers.

II.

A very important consideration is whether these substances represent toxic split products of the culture fluids produced by the bacterial growth, rather than toxic products secreted by the bacteria. In favor of regarding them as split products of the medium is the fact that they were more potent when the media was rich in proteins. Also suggesting this is their apparent non-specificity, in that, as will be shown directly, we obtained similar substances from many other organisms including non-pathogens. Again, in our experiments up to date, as will be seen, we have no reason to believe that they are in any sense antigenic.

Against the conception that they are split products of the constituents of the media are the following facts:

The substances were often high in potency from simple hormone media, as well as from media containing rabbit's blood and other proteins.

It seems that they are more potent in young cultures at the height of their growth energy. Potency seems to be at its maximum after 22 hours, decreasing with 48 and 72 hour incubation periods.

There is definite diminution of potency by filtration.

That there is a definite loss of potency by heating it 80° or below.

That there is a definite incubation time, rarely shorter than one hour, and never shorter than 40 minutes, even when large doses of relatively high potency are injected.

The incubation period which is characteristic of these "X" substances, differentiates them sharply from histamin and tyramin. Furthermore, the symptoms produced in rabbits by injections of ergamine differed distinctly from the symptoms produced by the "X" substances. The histamin when injected into rabbits in minimum lethal doses produced immediate acute death, and when injected in sublethal doses produced immediate sickness, followed by recovery, the animal usually being perfectly well after ½ hour. The supposition that the toxic action of the "X" substances might be due to substances analogous to histamin produced by the growth of the bacteria in the culture media, is thus rendered unlikely since the symptoms produced by the latter class of sub-

stances are immediate. The relatively greater susceptibility of rabbits to the "X" substances as compared to that of guinea pigs also excludes substances of the histamin class, since no such difference has been noted in experiment with histamin. Also histamin and other toxic amines when produced in cultures of bacteria are usually found in cultures some days older than those used by us. Chemical and pharmacological analysis of our poisons has not yet been undertaken.

III.

In order, further, to investigate the importance of split products of the medium, we attempted to produce the poisons with cultures grown on solid media. When the streptococci were grown in flat bottles on agar, chocolate agar, or Loeffler's medium for 20 or 22 hours, then washed up in salt solution, shaken for 2 minutes, filtered through Berkfeldt, and the filtrates injected intravenously into rabbits, symptoms exactly analogous to those produced by liquid culture products were observed, with the constant and definite incubation time and the same train of symptoms. In general, the solid media washing substances were less potent than the broth culture filtrates, but there was great irregularity and occasionally these filtrates killed acutely.

In all cases proper controls were made in order to eliminate agar-anaphylatoxin injury, a factor which was insufficiently controlled in similar experiments of Kraus, Kraus and Doerr, and Arima. This factor too is totally eliminated in those of our experiments in which we grew the bacteria on Loeffler's medium.

IV.

The symptoms produced in the animals treated with streptococcus preparations were entirely analogous, though less severe, as a rule, to those observed in parallel experiments carried out with the influenza bacillus in our laboratory by Mrs. Parker. In consequence, with Mrs. Parker and Miss Kuttner, we undertook a comparative study of the production of similar poisons with streptococci, typhoid bacilli, and influenza bacilli, and did a few isolated experiments with dysentery, meningococcus, pneumococcus and staphylococcus.

With Gram-negative bacilli like typhoid, we felt that we had to be particularly careful to eliminate the extractive substances spoken of as endotoxins. In consequence, we grew typhoid bacilli on various media for the shortest possible periods, consistent with good growth using filtrates of 4, 6, and 12 hour cultures. These always gave definite sickness in rabbits intravenously injected, when 4 or more cubic centimeters were administered, and in all cases the incubation time and the symptoms were in every way similar to those produced by streptoccus and influenza filtrates. Comparison of such young 6 hour culture filtrates with the filtrates of 6 and 10 day cultures showed that the 6 hour filtrates made the rabbits quite as sick within the first 2 or 3 hours as did the extraction posions from the old cultures. The symptoms were somewhat different, however, rabbits receiving those from young cultures usually recovering in a short time, whereas, the others went on to a typical endotoxin death.

We may say in passing that we do not believe it possible even with 6-hour growths to eliminate the presence of extractive substances because rabbits treated with 6-hour filtrates always developed agglutinins and complement-fixing bodies, a fact which shows that investigators who have worked with filtrates of 5- or 6-day cultures of typhoid and dysentery could never have worked with pure exotoxins. And the sera produced with such 5-day filtrates must have contained considerable amounts of sensitizer to the bacterial protein to which some, at least, of their always limited protective action must be attributed. This is rendered still more likely by the fact that such sera have been strongly protective only after incubation with the antigen in vitro before injection. In general, as far as we have gone, it has seemed that the filtrates of young typhoid cultures were very much more toxic for rabbits than for guinea pigs, being, in this, similar to streptococcus and influenza filtrates. Filtrates of old cultures on the other hand were relatively more toxic for guinea pigs.

As to deterioration of the 6-hour typhoid substances at room temperature, our results have been irregular, except that it has seemed that after 24 hours' standing, there was distinct loss of toxicity in isolated instances.

In one experiment there was marked deterioration of toxicity

after heating to 70° for 30 minutes, and in 3 other experiments heating to from 75° to 80° for 30 minutes almost completely destroyed the toxicity of 6 and 12 hour typhoid filtrates.

There was, thus, marked similarity between the influenza and the typhoid substances, in all these particulars.

With both of these organisms experiments were now done with growths on solid media.

Cultures on various solid media were allowed to grow 6 hours in the case of the typhoid bacilli, in order to prevent as far as possible any bacterial cell death. Filtrates from such washings regularly produced sickness analogous to that described above.

Although acute death was sometimes obtained, in general the filtrates of washings of typhoid cultures as young as these have not killed, producing only marked illness after the regular incubation time.

With the influenza bacillus it was necessary, because of the nature of the organism to grow the cultures on chocolate agar. The shortest period of growth of these organisms on solid media was 12 hours.

Isolated experiments were done with other bacteria. Toxic filtrates were obtained from a 23-hour meningococcus culture on horse-serum-hormone-broth; from a 5½-hour culture of *prodigiosus* on rabbit-serum-hormone-broth, and mild reactions were obtained with a similarly prepared 23-hour Shiga bacillus culture filtrate.

With solid media washings, we produced definite sickness after the ordinary incubation time, and death in 18 hours with a filtrate from the washings of a 20-hour *prodigiosus* culture on hormone agar, and 2 deaths (in 2 and 3½ hours, respectively) with filtered washings of 5½ hour *B. coli* cultures on similar media. Mild but definite illness followed in two cases after the injection of filtered washings of 5½-hour cultures of the Flexner bacillus on hormone agar.

In a few isolated experiments we failed to obtain any indications of the formation of the poisonous substances with staphylococcus and pneumococcus, Type I.

All attempts to establish specificity or non-specificity in immunological experiment by treating rabbits with filtrates of the

various organisms and testing cross protection, have failed because, so far, we have no evidence of antigenic action of these substances. Repeated injection of rabbits with doses too small to produce definite sickness that is, doses of 1 to 2 c.c., have resulted in emaciation, gradual falling out of hair, and eventual death of the animals by apparent chronic injury. In such experiments rabbit protein was used in the media to exclude anaphylactic injury. We have not yet been able to obtain any of these poisons in sufficient potency to permit us to draw conclusions from systematic experiments concerning their action after intraperitoneal and subcutaneous injection. Although we have obtained some indication of injury after subcutaneous injection this point calls for further study.

SUMMARY AND CONCLUSIONS.

It will be noticed that whatever the organisms used, whatever the medium, and whether the substances used were filtrates of liquid cultures or filtrates of washings of young cultures grown on solid media, the general nature of the toxic effects, incubation time, etc., were always the same. Autopsy findings in all such cases in which acute death was obtained, never showed lesions that might in any way be regarded as characteristically defining the pharmacology of the poisons. These facts, together with our failure to establish specificity by any immunological tests, inclines us to believe that in all cases the poisons were of a similar, perhaps of the same nature.

The fact that there was a regular incubation time, that the toxic substances seemed to show heat instability, that they had a relatively greater toxicity for rabbits than for guinea pigs, and that they appeared in young cultures with apparent diminution as the cultures grew older, constitute evidence which prevents our dismissing them, peremptorily as split products of constituents of the media.

We cannot, at the present time, characterize them definitely, but we are safe in asserting that they are neither exotoxins in the ordinary sense, and cannot be regarded as endotoxins or extractive substances from the bacterial bodies.

We believe that it is these substances which have been regarded by many writers as exotoxins of streptococci. Also, it is not unlikely that they represent the substances which have been

regarded as specific exotoxins by Kraus and his co-workers, and by Arima, who produced toxic effects in rabbits by the injection of the washings of typhoid and dysentery cultures grown on solid media. We have recently been told by Dr. Anderson also that in immunizing horses with agar cultures of meningococci, he has found it advisable to wash the cultures once in salt solution in order to diminish the toxic effects noticed in horses when this was omitted. Such observations also would be explained by a knowledge of the substances we have described. We may state that recently a horse turned over to Mrs. Parker by Dr. Park for immunizing with influenza culture filtrates, died after the nineteenth subcutaneous injection. These cultures were grown on horse-blood-chocolate broth, a fact which should exclude ordinary anaphylactic effects.

We have worked with these substances for considerably over a year, making many cultures in many different ways and using several hundred rabbits, and have found that there are great experimental difficulties which make it impossible for the present to define the biological properties of these substances with the accuracy which can be applied to more potent substances such as the true toxins. We have no experimental evidence to show that these "X" substances are produced by streptococci, or other bacteria, during their growth within the animal body. The condition of rabbits injected with cultures at a time when general septicemia ensues some hours before death, is strikingly similar to that which is produced one or two hours after injection with the filtrates, and the filtrates produce a leukopænia similar to that which occurs as the body is being overwhelmed by the streptococcus infection. However, the low potency of the poisons and other difficulties of working with them, makes it impossible at the present time to approach this problem more closely.

We cannot, therefore, at the present time do anything more than submit the observed facts with the hope that experimental difficulties may be overcome in the future.

Such as they are, however, these "X" substances are very definite and probably non-specific products which appear early in cultures and with regularity, and which must be taken into consideration in all work in which bacterial cultures or their derivatives are injected into animals.