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PREPARATION OF A LOW-CHLORIDE MEDIUM, FOR THE PRODUCTION OF DIPHTHERIA TOXIN.

«AN ADAPTATION OF HOLT'S TECHNIQUE»

by

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During the period elapsing between 1940 to 1951, diphtheria toxin, used to be produced in Iran, in a medium based on peptic digest of meat (1), for immunoligal purposes.

Later, Muller's medium, as modified by Holt (2), based on high-chloride acid hydrolyzate of casein was used for large scale preparations (3).

Casein hydrolyzate mentioned above, is usually prepared by using 3-8 Normal hydrochloric acid as hydrolyzing agent; and by distillation of this product under vacuum, it is only possible to reach a Na :Cl ratio of 1 :1. Therefore the addition of 36% sodium hydroxide as a neutralizing agent into the acid hydrolyzate, simply elevates the salt concentration of the solution, thus producing a limiting factor, for the production of diphtheria toxin in the medium.

For this reason, preparation of a low-chloride medium for cultivation of C. diphtheria, has been the ultimate goal of many workers in this field (4-6).

To overcome the inhibitory action of the salt present in the medium, some investigators have been forced to lower the nitrogen content of the medium below an optimum (6). Several others have removed excess Hcl, by repeated distillation of the hydrolyzate, and as much of the remaining Cl as possible, either with lead oxide or with commercial "Dry white lead" (5-6). Few others have used a mixture of HCl and H_2SO_4 as hydrolyzing

agent, removing the excess sulfuric acid with $Ba(OH)_2$ (5).

Difco Laboratories, Inc., Detroit, Michigan, supplies the low-chloride product, under the trade name "Casamino Acids", and the high-chloride as "Casamino Acids, Technical", and the way in which the low-chloride product is prepared, is unknown.

The present paper deals with three experimental procedures concerned with the preparation of a low-chloride medium that could be used for large scale productions.

EXPERIMENTAL

The medium used in this study was originally that of Mueller's (8-10), modified by Holt (2), and adapted by Mirchamsy et al. for diphtheria toxin preparation (3).

Using a high-chloride medium, an average toxin titre of 70 Lf./ml. has been obtained. Followings are some minor modifications of our earlier technique (3), and the methods used for lowering the salt concentration of the medium, and obtaining a higher titre of the toxin produced.

Protein Hydrolyzate

As it was previously stated, using a high-chloride medium, an average toxin titre of only 70 Lf./ml. could be obtained. In order to increase the titre, it was decided to bring the salt concentration to as low an amount as possible, by (a), using a more dilute acid (2N) so as to leave a lower acid concentration in the hydrolyzate, (b) using lead oxide for removal of excess chloride, and (c); by using an ion exchange resin, for removal of excess HCl, left in the hydrolyzate :

Two different mixtures of casein * in HCl solutions were made in two 12-litre Round Bottom Pyrex flasks, each containing one kilo casein in five litres of HCl solution. The difference was in concentration of the acid solutions, being 2 and 3 Normal in the flasks, successively.

The flasks were both gently boiled in two heating jackets, and then refluxed for 20 and 16 hours, respectively.

The blackish solutions thus obtained were distilled twice under vacuum, until no more acid distilled off. To each black viscous distillation residues, were then added; two litres of boiling distilled water, and the flasks were shaken, until the residues dissolved.

Into each lot, 36% sodium hydroxide (ANALAR) was added to bring the reaction to pH 5, after which they were mixed with decolourizing

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* *BDH, Light, White, Soluble*

charcoal (* *) and left overnight at room temperature.

To the decolourized yellowish filtrates, 10 gm. pure, iron free CaCl2 (per kilo casein, primarily used) were added, and the one corresponding to 1 kilo casein hydrolyzed with 3N HCl, was divided into two lots, each being treated as follows :

The first lot was primarily treated with lead oxide, and the excess Pb was removed first with H_2SO_4 then with BaS, and finally the Ba removed with H_2SO_4 so as to leave a very low concentration of the latter present. The solution was then neutralized with 36% NaOH.

This was carried out, exactly as discribed by Mueller (6).

The second lot was passed through a large chromatographic column filled with Amberlite IR-4B, prepared and pretreated as stated by C.H.W. HIRS et al (7). This was collected in a beaker, and defurruginization on three samples was carried out by Mueller's method. (8).

The defurruginized solutions were then analyzed, for their Nitrogen, Iron, and Chloride contents.

PREPARATION OF THREE 10 - LITRE LOTS OF MEDIA, THAT HAVE ALSO BEEN SIMPLIFIELD FOR LARGE SCALE PREPARATIONS:

Into 3 marked 10-liter containers were added :

Dist. water	5,000 ml
Mueller's solution II (8)	25 "
10%, Cystine solution	37.5 " *
Lactic acid (ANALAR)	25 " **
Maltose (Difco)	150 gm
Ca Cl2 (dried)	1.2 "

Stock hydrolyzate solutions; necessary amount from each, to give a final nitrogen content of O.11 per cent.

Dist. water, to make	10,000 ml
PH	6.9

The media were dispensed in Roux boxes, autoclaved and then inoculated with P.W. 8 strain. * * *

** *Darco, G-60*

* & ** *These two have recently been increased and decreased correspondingly by Holt.*

*** *This has generously been supplied by Dr. Holt, from the Wright Fleming Inst. London.*

RESULTS

Method used for casein hydrolyztae preparation	Lf./ml.	Kf./min.
Using PbO technique.	83	1
Using 2N HCl.	83	1
Using Amberlite IR-4B.	115	1/2
Using High-Chloride hydrolyzate.	75	1

As it is apparent from inspection of the table, the use of Amberlite ion exchange resin, leads to a high toxin titre as well as a low chloride medium, although it is not a time consuming technique.

Discussion

Using a 2N hydrochloric acid as hydrolyzing agent, followed by repeated distillation of the hydrolyzate, it was only possible to lower the salt concentration of the medium to an amount which still was above the optimum level of 0.5%, in the final preparation.

Removal of excess chloride from protein hydrolyzate, by the time consuming lead oxide technique, led to a low-chloride hydrolyzate, but it did not give a good result from toxin titre point of view. (In the paper presented by Mueller (4), it is claimed that using the latter technique, together with "shallow layers" of the medium, the yield of diphteria toxin has been increased from 60 to ABOUT 100 Lf./ml.).

As it is noted from the table of the results, ion exchange technique stands more satisfactory, from the view point of simplicity and increase in toxin titer.

It is to be reminded that, in order to eliminate bacterial contamination, and to save time, maltose was directly added to the medium, before being autoclaved; and the reaction of the medium was adjusted to pH 6.9.

It has been proved that, the use of a filtered or even separately autoclaved maltose solution, yields still a higher toxin titre in the medium already discussed.

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SUMMARY

Attempst have been made, to lower the salt concentration of the medium, to the optimum level of 0.5%, in the final preparation.

Dilute HCl, Lead oxide, and Amberlite IR-4B have been separately used, and the latter has led to a toxin titer of 115 flocculating units per c.c.

By the way, preparation of a simplified medium, for large scale diphtheria toxin pruductions is briefly discussed.

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