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HORSE SERUM PURIFICATION RESIDUE AS A NITROGEN SOURCE FOR THE GROWTH OF MICROORGANISMS. II – CORYNEBACTERIUM DIPHTERIA (TOXIN PRODUCTION)

by

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Introduction

The large scale production of diphtheria toxin in synthetic media for the preparation of potent toxoids without side effects in man, is of considerable importance and is a routine work of most prophylactic manufacturers.

(Pappenheimer and Johnson 1936) were the first to prepare such a medium for diphtheria toxin preparation.

(In 1937 Pappenheimer et al) proposed a synthetic medium composed of several amino-acids as the source of nitrogen. (Mueller et al 1939) used casamino-acids (a commercial product of acid hydrolysate of casein) as the source of nitrogen for large scale production of diphtheria toxin.

This medium is already used by many toxin manufacturers. (Holt 1950) developed techniques for acid hydrolysis of casein and described a formula for toxin production.

The Holt technique has been applied with minor modifications at Razi Institute since 1954 and is used for large diphtheria toxin production.

The purpose of this note is to describe a new formula containing acid hydrolysate of horse albumin and other proteins normally remaining after the purification and concentration of therapeutic sera according to the technique developed by (Pope et al 1939).

Materials and Methods

Preparation of the medium.

a) The protein Hydrolysate.

The Horse Serum Residue obtained after removal of ammonium sulphate as

described previously (Manhouri, Ardehali 1966) was hydrolysed by applying the method of Holt (1950) modified by (Spies 1952) as follows:

To five kg of Residue (containing 20 per cent protein) in a 12-liter pyrex flask add 1800 ml of concentrated HCl. The mixture was gently heated under a reflux condenser for 10 hours. The black solution was then distilled under vacuum to reduce its acidity. The black viscous residue was dissolved in 1-liter of distilled water, 10 g pure calcium hydroxide was added and the pH was adjusted to 5.0 by adding 36 per cent NaOH solution to the mixture.

To the cold mixture, 200 g charcoal (Darco G 60) was added, left overnight, and filtered the next day.

To the decolorised filtrate, 12,1 g of anhydrous CaCl2, 104 g Na2HPO4, 7H20 and 57 g KH2PO4 was added for each kg of original protein. The PH was then adjusted to 7.6 with 36 per cent NaOH and heated for 10 minutes at 80°C.

The hot filtrate was then filtered through iron-free filter paper and when cold kept at 4°C under chloroform.

This stock hydrolysed solution of protein residue contained/: Amino-Nitrogen 17.78 mg/ml. Total nitrogen 18.4 mg/ml, Sodium Chloride 80 mg/ml and ratio of NaCl/Amino-N 4.4.

b) Preparation of the medium (1 liter).

Protein hydrolysate	ml equivalent to 1.1 g amino-nitrogen
Solution II Mueller (Holt 1950)	2.5 ml
Solution l-cystine (10 per cent)	3.75 ml
Lactic acid (90 per cent Merck)	2.5 ml
Maltose Difco	15 gr
Anhydrous CaCl2	0.120 gr
Distilled water	up to 1000 ml

To 500 ml distilled water, the required amount of filtrate, Mueller solution, l-cystine solution, lactic acid, maltose, and anhydrous CaCl2 were added. The volume was then brought to 1000 ml and the pH was adjusted to 6.9 with 36 per cent NaOH. The medium was dispensed in 160 ml volume into 1 liter pyrex Roux bottles and autoclaved at 110°C for 20 minutes.

c) Toxin production.

1—Strain: The strain P.W.8 (G12/6) received from Dr. Holt (1956) was used throughout the investigation.

2—Preparation of subcultures: A growth of the above strain in Roux bottles of 1 liter containing 160 ml of media was used as the subculture.

3—Seeding of batch: The batch was inoculated by the above subculture (1 ml per bottle containing 160 ml of medium) and was kept at 33-34°C for a period of 6 days.

4—Harvest of toxin solution: The culture was filtered after 6 days, first on hard filter paper and then on EKS sterlizing pads.

d) Titration:

1—The floculation test was done according to (Ramon 1922).

2—Minimal lethal Dose was determined by inoculation of 1 ml of dilutions of toxin into 250 g. guinea piegs.

3—Protein nitrogen determination: The Kjeldahl method with precipitation of proteins by trichloracetic acid was used.

Results

The results of 10 batches of 20 liters are summerized table 1. The maximum amount of toxin obtained was 73 Lf/ml and the maximum purity was 1235 Lf/mg. N. P.

Table I: Production of diphtheria toxin in horse serum residue (acid hydrolysis)

Batch No	Fe++ added to R. bottle 160 ml gamma gr.	Lf/ml	Kf/min.	M.L.D/ml	Lf/mg. N.P
1	5	58.5	2½	N.T*	886
2	2.5	68.5	2	N.T	1104
3	5	49	2½	5000	731
4	5	53.5	3	N.T	922
5	7.5	68.5	2	8000	992
6	5	49	2	N.T	890
7	—	57	2	6.000	1055
8	2.5	63	2	8000	1235
9	—	63.5	1	8000	920
10	—	73.5	½	8000	1065

* N.T = Not tested

Discussion

The preliminary results shown in this note are encouraging since the toxicity of toxins obtained in this medium was similar to that of Holt's medium. Work in

progress indicates that with some modification, Lf/ml and the purity of the toxin obtained may be increased to the same level as medium prepared with acid hydrolysate of casein.

The antigenicity of toxoids prepared with this medium was quite satisfactory and will be the subject of another publication.

Summary

An inexpensive medium based on acid hydrolysis of by-products of therapeutic sera is described for the production of diphtheria toxin.

The preliminary results indicated that the toxin obtained was highly toxic and may substituted the toxins produced in semi-synthetic media.

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