Concentration and Purification of Diphtheria Toxoid by Means of Sulphosalicylic Acid for the Preparation of Diphtheria Prophylactic

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The possiblity of using sulphosalicylic acid for the precipitation of diphtheria toxoid was examined. It was found that under certain conditions this acid may be used with satisfactory results. The diphtheria toxoid precipitated with sulphosalicylic acid and further purified with ammonium sulphate and adsorbed on aluminium phosphate, yielded a product which satisfied all requirements for a good diphtheria prophylactic.

A considerable number of methods, using inorganic and organic acids, have been published for the precipitation of diphtheria toxoid.[1–10]. All of them yield more or less satisfactory results. The main objection to their use is the possibility of destruction of the antigen which is indicated by prolongation of the flocculation time (Kf).

It is also known that even methods which do not use acids, for instance precipitation with alcohol[11] or combined methods, do not always give satisfactory results.

These facts encouraged us to investigate a classical agent for the precipitation of proteins, such as sulphosalicylic acid (SSA).

MATERIALS

The investigation was carried out on three different diphtheria toxoids obtained from three different media: papain broth,[12, 13] casamino-hydrolisate medium[14] and Pope's tryptic-digest medium[15]. The strains used were: for the papain and tryptic-digest media a strain derived from *Corynebacterium diphtheriae* PW 8, CN 2000 (Wellcome, London); and for the casaminoacidshydrolisate medium a strain from Massachusetts's Department of Public Health*. The detoxication was carried out by adding 0.55% of 40% formaldehyde solution and keeping the toxoid at 34° for six weeks. The innocuity test was performed on guinea pigs by the intracutaneous and subcutaneous routes.

The flocculation test, according to Ramon[16], was carried out with our substandard made from a purified diphtheria antitoxin which flocculated with crude toxins within 5—6 minutes and with crude toxoids within 6—8 minutes in a water-bath at 45°.

Diphtheria toxin or toxoid and diphtheria antitoxin precipitate each other in optimal proportion. To a series of tubes increasing quantities of antitoxin of a known potency, expressed in antitoxin units (AU) are placed. To each tube the same

* Obtained through the courtesy of Dr. Tasman, Rijks Instituut vor de Volksgezondheid, Utrecht, Holland.

quantity of toxin or toxoid is then added. The tubes are placed in a water-bath at 45—50°. Turbidity occurs in the majority of the tubes with increasing intensity. In the tube with the optimal proportion of antigen and antitoxin flocculation occurs. The potency of a toxin or toxoid obtained by this method is expressed in Lf units (Limes flocculationis). The time Kf, expressed in minutes or hours, within which the flocculation ensues is also of a certain value, for it indicates that the antigen has remained unchanged in its ability to react with the antitoxin, consequently that it has not undergone any destruction during various operations.

The pH values were measured with a Beckman Glasselectrode pH meter, model H2. The turbidity was checked by Fisher's Electrophotometer, with red (650) and green (525) filters. The total nitrogen (TN) and protein nitrogen (PN), was estimated with a macro-Kjeldahl apparatus.

The abbreviations Lf / mg. TN and Lf / mg. PN mean the ratios between Lf units and the amount in miligrams of total nitrogen and protein nitrogen found by Kjèldahl's method. By these terms the »purity« of the toxin or toxoid crude or refined is expressed.

For the precipitation 25 or 50% w/w of an aqueous solution of sulphosalicylic acid* was used. To 100 ml. of toxoid 0.5—2.0 ml. were gradually added and the pH and extinction values E were checked.

For the centrifugation a Sharples Supercentrifuge Laboratory type with the clarifying bowl was used. The out-flow speed was regulated to about 7 liters per hour, so that a clear supernatant was obtained. Further details will be given in the text .

METHODS AND RESULTS

With gradual addition of sulphosalicylic acid to the toxoid the turbidity from the formed precipitate increases, whereas pH values decrease. Before measuring the turbidity it is necessary to shake the samples in order to prevent sedimentation.

Fig. 1 shows the precipitation curve, constructed by recording the pH and turbidity values. Fig. 2 demonstrates the precipitation curves of another papain broth toxoid, a sample of casamino acids medium toxoid and a sample of Pope's tryptic digest broth toxoid. These media-differ considerably in their content of non-specific proteins. The most inferior in this sense is the papain broth toxoid which contains much non-specific protein. This is obvious from Fig. 2 (compare the extinction values). Further information is given in Table I where the chemical and other characteristics of these toxoids are recorded.

From Fig. 2 it may be seen that precipitation was completed practically at pH 3. The precipitation was carried out with 25% sulphosalicylic acid, adding 2 ml. quantities each time to a sample of 100 ml. of toxoid. The extinction was measured with a green filter B (525).

The sample of toxoid is totally precipitated at pH 3, then centrifuged and dissolved with distilled water by adding a 2% ammonia solution. The sediment of the papain broth is at this pH of a dirty greyish-yellow colour and fragile consistence and must be stirred by means of a glass rod (or, still better, an artist's brush). The clear, yellowish solution, is brought to its original volume, and pH adjusted to 7.5. The solution is then examined for its number of Lf units per ml. by means of the flocculation method. Generally, 85—90% Lf of the original sample is found. We were also interested in the content of Lf

* Analytical grade reagent »Pliva«.

units at various pH. Two such examinations are shown in Table II. It was noticed that the colour of the sedimend grew more and more dirty pink with increasing pH.

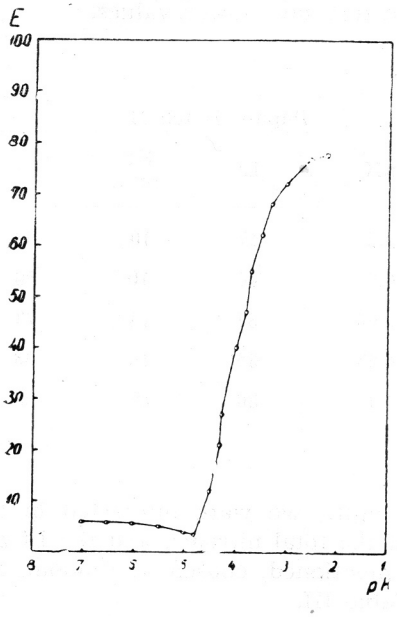

Fig. 1.

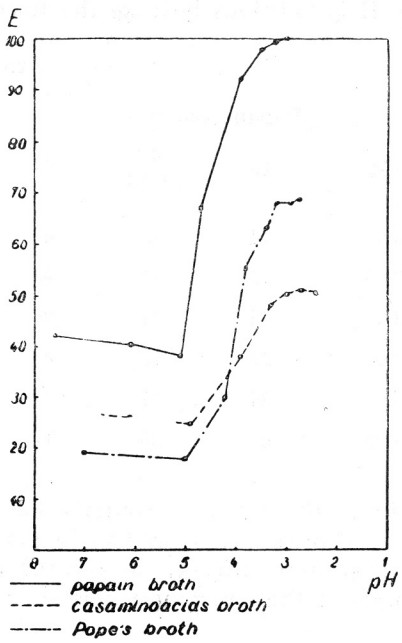

Fig. 2.

Fig. 1. Precipitation curve of a papain broth toxoid.
Fig. 2. Precipitation curves of toxoids derived from three different media.

TABLE I.

Medium	Lf/ml.	Kf min.	TN mg./ml.	Lf mg.TN	PN mg./ml.	Lf mg.PN
Papain batch I	51	8	3.192	15	0.0735	693
Papain batch II	51	7	3.325	15,4	0.08	637
Casamino batch II	52	6	2.429	22	0.042	1285
Pope batch 4 & 5	44	9	1.932	22	0.033	1333

With the change in colour of the sediment the colour of the supernatant fluid grew more and more red. It is probable that this red colour is derived from porphirin which influences the quality of the final product.

From Table II the impression is obtained, that the lower is the pH the larger the content of Lf. However, this is only partly correct. From the first example in this table, and from other examples which are not shown, the fact emerges that with an acidity over pH 2.5 the number of Lf units begins to decrease again, while the Kf is prolonged. This may be attributed to the

destruction of antigens by the great acidity. This phenomenon has also been noticed with the earlier mentioned precipitation methods using various inorganic and organic acids. The prolongation of the Kf at higher pHs recorded in Table II is fictitious beacuse the flocculation test gave lower values.

TABLE II.

Papain Batch I				Papain Batch II			
pH	Lf	Kf min.	%	pH	Lf	Kf min.	%
3.0	44	13	86	3.2	47	10	92
3.25	46	11	90	3.6	44	10	86
3.5	45	11	88	4.05	37	14	73
3.75	42	10	81	4.25	35	16	68
4.0	34	17	63	4.4	30	15	59
4.25	31	39	60				

In addition to the contribution in Lf units, we were interested in the degree of purity expressed in Lf per mg. of the total nitrogen and the Lf per mg. of protein nitrogen at the pH points mentioned, chosen at random. An example of this examination is shown in Table III.

TABLE III.

	pH	Lf	%	Kf min.	TN mg./ml.	$\frac{Lf}{mgTN}$	PN mg./ml.	$\frac{Lf}{ngPN}$
Papain Batch I	4.5	20	36.5	31	0.0665	301	0.045	444
	4.25	36	67	8	0.07	514	0.0385	945
	4.00	40	74	5	0.07	557	0.045	866
	3.75	43	80	5	0.084	512	0.054	798
	3.25	45	83	5	0.0875	502	0.061	721

From Table III it is seen that the best »purity« of the precipitate was found at a pH between 4.5 and 4.25. Further, it may be seen that the optimal contribution in Lf units does not tally with this »purity«. It should be pointed out, that the samples, after being precipitated, centrifuged and diluted, were dialysed in tap water for two days in order to eliminate all sulphosalicylic acid. A 5% ferric chloride solution was used as reagent for sulphosalicylic acid. Sulphosalicylic acid in higher concentrations somewhat hinders the flocculation by prolonging its duration, and possibly damages the antigen after a prolonged standing. During all our experiments under these conditions no serious damage of the antigen was observed.

EXPERIMENTAL

On grounds of these preliminary examinations we tried to prepare a vaccine with an antigen purified in this way and to test its validity by applying the methods generally used in immunology. With this aim in mind we prepared several series of purified diphtheria vaccine. All these series were submitted to every required test and yielded results which fulfilled all criteria of modern, adsorbed diphtheria vaccines.

The following is a description of the method of producing diphtheria vaccine as applied in our Laboratory now, by means of toxoids with all three media, namely papain medium, casamino medium and the medium as per Pope.

I. *Papain diphtheria toxoid batch* II.

Volume 263 liters, titre 51 Lf. Kf 7 minutes. TN 3.325 mg./ml.; Lf/mg. TN 15.4; PN 0.08 mg./ml.; Lfmg./PN 637; pH 7.63. Total amount of Lf units = 15.413,000 (for precipitation curve see Fig. 2).

Ten liters of toxoid are poured into a 20 liter enameled pot and by means of a separatory funnel a 25% solution of sulphosalicylic acid is slowly added and stirred until the pH 4 is obtained (about 400 ml., i. e. 1% was required approximately). Within a few minutes, large, rough floccules are formed and thereafter sedimentation sets in. This mixture is now poured into a tubus flask, lifted some 2 meters high, and from this the contents are allowed to flow freely into a Sharples supercentrifuge at about 25,000 rpm. A 19 × 14 cm celluloid sheet is put into the clarification bowl in order to facilitate the removal of the precipitate.

After 20 liters have passed through the centrifuge, the latter is stopped, the bowl taken out, and the sediment scraped off the celluloid sheet by means of a spatula. This sediment is of a dirty pink colour, fragile and insoluble in water. Therefore it is first ground in a porcelain grinder by slowly adding ammoniacal water (2 ml. of 2% ammonia in 200 ml. of distilled water) until a homogenuous liquid pulp is formed. The remaining particles which could not be ground with a porcelain rod are easily crushed by means of an artist's brush. Then a 2% ammonia solution is added drop by drop with careful slow mixing. After a certain amount has been added the sediment begins to dissolve, as may be seen by the change of colour to an intensive red. This operation ought to be carried out very quickly since the sediment on the brink of the porcelain jar, the celluloid sheet and the grinding rod is drying fast, whereby the dissolution is hindered and consequently the yield diminished. The contents are then rapidly emptied out of the grinder into a Jena beacker and put on a magnetic mixer where the pH is adjusted to 7.5—7.8. The remaining clumps are crushed with a brush and mixed for half an hour. The concentrate obtained in this way is then dialysed against tap water for two days, either in a cellophane bag or cellophane tubing. After two days we test the presence of sulphosalicylic acid by examining a dilute sample (1 cc of concentrate + 4 cc of distilled water + a few drops of a 5% ferric chloride solution). The concentrate, to which merthiolate 1:5000 is added, is now poured into a bigger flask where all the series of toxoid destined for the purification process are collected. The concentrate is of a raspberry-juice colour. In such a way 40 liters of toxoid are concentrated daily. The collected dialysate of over 263 liters of toxoid amounted to 4250 ml. with 2750 Lf/ml., Kf 6 minutes, with a total of 11.687,000 Lf, i. e. a yield of 76 per cent of the original toxoid. Kjeldahl determinations showed the following values for nitrogen: TN 3.616 mg/ml; Lf/mg. TN 753 (crude toxoid 15.4); PN 3.476 mg./ml.; Lf/mg. PN 790 (637). In the first stage of the purification process a partial purification is achieved and this is shown by the purification factor:

$$\frac{\text{PN of concentrate}}{\text{PN of crude toxoid}} = \frac{790}{637} = 1{,}25.$$

The further purification process consists of a two-graded salting out of the antigen with ammonium sulphate.

Two methods are applied: the one suggested by Holt[17] or another by Tasman. Holt, in the final stage of his purification process salts out the antigen first with one third of the saturated ammonium sulphate. This addition of ammonium sulphate leads to partial precipitation of proteins which are considered to be mainly non-specific. After filtration through filter paper the sediment is discarded and solid

ammonium sulphate is added again to the filtrate up to a two thirds saturation. Precipitation occurs again, with the difference that a big mass of protein is precipitated this time. We filter once more through filter paper, collect and dialyse the sediment, and after the dialysis we add merthiolate 1:10,000, then filter under sterile conditions through a Seitz-filter. The purified material thus obtained is now adsorbed on aluminium phosphate.

Another method of purification or salting out by means of ammonium sulphate is Tasman's method[18,19].

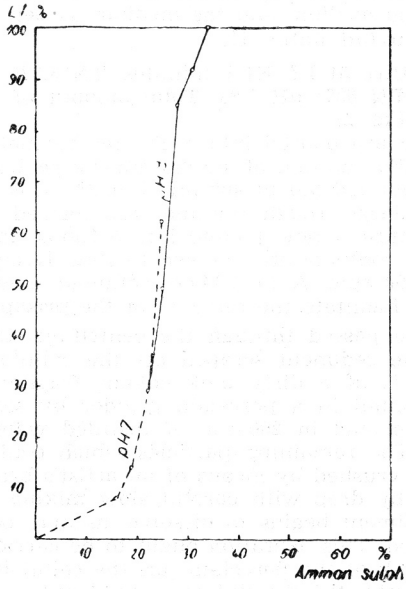

Fig. 3. Salting out with ammonium sulphate at pH 7 and pH 8

In this method an amount of ammonium sulphate, previously determined experimentally for lower and upper limit of salting out is added to the concentrated antigen after ultrafiltration. This procedure may be illustrated graphically by platting the percentage of ammonium sulphate on the abscissa, and the obtained Lf values on the ordinate[18, 19]. Thus a curve is obtained, or better two half-curves, the one at pH 7 and the other at pH 8. The first has a curvature at the beginning and the second at the end. A good purified product is obtained if that amount of ammonium sulphate is used which cuts the curvatures of the mentioned curves, i. e. if for the purified product the straight part of the curve is used (Fig. 3 shows such a graph).

We treated in this way our dialysate after precipitation with sulphosalicylic acid. The dialysate amounted to 4,250 ml. According to Holt it ought to be diluted to about 1,500 Lf per ml. i. e. 3,750 ml. of distilled water had to be added to our dialysate to bring its volume to 8,000 ml. To this half the volume of a saturated solution of ammonium sulphate, (760 g of solid ammonium sulphate per 1 liter of water at 20°) i . e. 4,000 ml. was added. The total volume was now 12,000 ml. After filtration through filter paper 11,250 ml. of a yellowish-brown liquid remained. (The dirty pink coloured sediment on the filter paper was discarded). In the second addition of ammonium sulphate 250 g. of solid ammonium sulphate per 1,000 ml. of filtrated product are added, i. e. 2.82 kg. of solid ammonium sulphate in total. After filtration through filter paper (overnight) the sediment was collected into a cellophane bag and dialysed. The filtrate was a light-yellow liquid in which a slight precipitation still occured when ammonium sulphate in crystals was added again to full saturation. The dialysis lasted some three or four days, as long as there

is ammonium sulphate in the dialysate, which could be determined by means of a 5% solution of barium chloride. The volume of the purified material after the dialysis amounted to 2,700 ml. with a titre of 3,200 Lf per 1 ml., and the Kf 9 minutes. The yield with respect to the crude toxoid amounted to 56.8%, and in proportion to the concentrated toxoid after the sulphosalicylic acid to 74.5%. Total Lf 8.740,00; TN 2.613 mg./ml; Lf/mg. TN 1,226; PN 2.38 mg./ml.; Lf / mg. PN 1,344. Accordingly the purification factor was $\frac{1,344}{637} = 2.1$, this being a satisfactory result.

By the same procedure casamino and Pope toxoids were purified also. It should be pointed out that the colour of the sediments, after they had been centrifuged on a Sharples centrifuge, was not dirty pink but greyish-green. When dissolved, their colour was respberry red, though not so intense as with papain toxoid.* Table IV shows all important data on the course of purification of these two toxoids.

TABLE IV.

Phase	Volume ml.	Titre Lf	Kf min.	Prec. at pH	TN mg./ml.	Lf / mg.TN	PN mg./ml.	Lf / mg.PN
Pope Batch Series 4 and 5								
Crude	204,000	52	6	3.5	2.429	22	0.042	1,285
Conc.	2,000	3,800	9	—	—	—	2.66	1,428
Purif.	1,700	4,000	5	—	2.4	1667	2.184	1,831
Sasamino Batch II								
Crude	78,000	44	9	1.932	3.6	22	0.0385	1,131
Conc.	800	3,400	8	—	—	—	—	—
Purif.	420	4,410	10	2.45	—	1800	2.38	1,850

Casamino Batch II:

Total Lf of crude toxoid: 10.600,000
Concentrated: 7.600,000; yield: 71.6%
Purified: 6.800,000; yield with respect to
crude toxoid: 62%
concentrate: 90%

Purification factor: $\frac{1,831}{1,285} = 1.42$

Pope Toxoid Batch Series 4 and 5:

Total Lf of crude toxoid: 3.440,000
Concentrated: 2.700,000; yield: 79%
Purified: 1.760,000; yield with respect to
crude toxoid: 51%
concentrate: 65%

Purification factor: $\frac{1,850}{1,131} = 1.63$

* Consequently these toxoids have less porphirin pigments than papain toxoid.

Adsorption on aluminium phosphate.

To complete the diphtheria vaccine in form of an adsorbate, aluminium phosphate prepared by the method described by Holt[20] is used as adsorbent. The adsorbent thus prepared contains 10 mg. of aluminium phosphate in 1 ml. One millilitre of our final vaccine should contain 30 Lf of purified antigen. In adding the purified antigen to the sterile suspension of aluminium phosphate and in the further process of completing the preparation of the vaccine with respect to the adjustment of the pH, the buffer, etc., we keep strictly to the technique described by Holt or Tasman, and recommended at the Conference of the Heads of Laboratories Producing Diphtheria and Pertussis Vaccines, 1953.[21]

The final vaccine prepared in such a way is a snow-white, fast sedimenting suspension; the supernatant fluid is transparent and colourless liquid which, in this respect too, satisfies the requirements of the modern prophylactic.

TABLE V.

Preparation	Titration (Date)	AU	Titration (Date)	AU	Titration (Date)	AU
Dipuran Series 4	Dec., 14, 1953	4.2	March, 5, 1954	2.35	Jan., 17, 1955	3.33
Dipuran Series 5	Jan., 13, 1954	3.5	Jan., 17, 1955	3.13		
Dipuran Series 6	Feb., 27, 1954	2.55	Jan., 17, 1955	1.97		
Dipuran Series 7	March, 27, 1954	4.8	Jan., 17, 1955	3.27		
Di-Te mixed vacc. Series 4	Nov., 22, 1954	3.142	Dec., 12, 1954	3.386		

In all the series of diphtheria vaccine prepared by the described method and tested so far, we could not find any free antigen in the supernatant either by flocculation or by a reagent for proteins (sulphosalicylic or trichloracetic acid), i. e. the antigen is completely adsorbed on aluminium phosphate. This may be proved by dissolving aluminium phosphate with sodium citrate. A vaccine sample is centrifuged, the supernatant poured off and 10% of sodium citrate in crystalline form and water are added to the precipitate. This is kept in an incubator for two days until the aluminium phosphate dissolves; water is then added to make up the volume of the final vaccine and flocculate. In such a way the total amount of the added antigen has always been detected. The flocculation time is somewhat prolonged.

In Table V the results are shown of the tests carried out so far with the series of diphtheria vaccine, Dipuran, and with one series of a mixed diphtheria-tetanus vaccine, Ana Di-Te. A group of five to ten guinea pigs were given a single injection of half a human dose (0.5 ml.), and after one month the amount of antitoxic units in the blood of each animal was determined separately. Thereafter the average value or the titre of pooled animal sera was determined. A good preparation ought to give an average value of at least two antitoxic units per ml. of guinea pig serum.

In the future we expect far better results from toxoids derived from casamino acid medium, production of which we shall start soon.

CONCLUSIONS

In this paper we have described our investigations on the use of sulphosalicylic acid for the precipitation of diphtheria toxoid, in order to employ this technique for routine production of diphtheria prophylactic. The results, obtained so far, show that this method provides quite a good vaccine. This

method is not intended to be proclaimed as the best one. The author's aim was only to include this method among others of the same value and to point out its simplicity, quickness and cheapness. We hope, that field trials will provide results as good as those obtained in animals.

The results of the field trials and the results of comparison of this method with other methods will be the subject of another paper.

In addition it should be mentioned that a sulphosalicylic acid precipitation of tetanus toxoid without further purification process may also be satisfactorily applied. The completed vaccine prepared in this way with 10 Lf/ml. and 800—1,000 or more Lf/mg. PN yielded an average of 4—8 A. U. per ml. in animals.

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IZVOD

Koncentracija i purifikacija difteričnog toksoida (s pomoću sulfosalicilne kiseline) za proizvodnju cjepiva protiv difterije

M. Stanić

Poznat je već cijeli niz metoda za precipitiranje difteričnog toksoida, kod kojih su upotrebljene različite anorganske ili organske kiseline. Tim metodama dobiveni su manje ili više dobri rezultati. Glavni prigovor odnosio se na mogućnost oštećenja antigena, što se očitovalo u produljenju flokulacionog vremena.

Mi smo se u svojem laboratoriju pozabavili ispitivanjem precipitiranja difteričnog toksoida sulfosalicilnom kiselinom i mogućnošću njezine primjene za proizvodnju cjepiva protiv difterije. Kod toga ispitivanja dobiveni su sasma dobri rezultati.

Za precipitiranje upotrijebili smo 25 do 50%-tnu sulfosalicilnu kiselinu. S pomoću Fisherova fotometra promatrali smo proces precipitacije, te smo na osnovu praćenja promjene pH i ekstinkcije, konstruirali precipitacionu krivulju. Ta krivulja pokazuje, da je precipitacija bjelančevina difteričnog toksoida završena oko pH 2,8. Precipitacija od pH 4,25 do 3,0 daje 60—90% Lf nativnog toksoida. Tom precipitacijom postiže se, pored koncentracije, i neki stupanj purifikacije. U rutinskoj produkciji, koju smo uveli, obično precipitiramo kod pH 3,5, pa onda centrifugiramo centrifugom Sharples kod 25.000 okretaja. Precipitat je u vodi netopljiv, pa ga otapamo 2%-tnim amonijakom. Kod pH 7,5—7,8 stavljamo otopljeni koncentrat u dijalizu.

Proces purifikacije sastoji se nadalje u isoljivanju amonijevim sulfatom — prema Holtovoj ili Tasmanovoj metodi. Dobivena čistoća, izražena u Lf jedinicama na mg. proteinskog dušika (Lf/mg. PN), iznosi 1300—1600 za papainski toksoid, no mnogo se bolji rezultati dobivaju kod toksoida dobivenih od Popeova bujona ili od podloge priređene od kazeinskog hidrolizata. Kod tih toksoida dobili smo vrijednosti i veće od 1800/mg. PN. Vrijeme flokulacije, Kf, ostalo je, prema našim dosadanjim iskustvima, otprilike jednako kao kod nativnih toksoida: oko 10 minuta. Ukupni je prinos Lf jedinica — u usporedbi s prinosom kod nativnog toksoida — oko 50—60%. Dobiveni purifikat adsorbiran je zatim na aluminijev fosfat priređen prema Holtu. Dovršena vakcina sadrži 30 Lf u ml. i 10 mg. $AlPO_4$ na 1 ml.

Serumi zamoraca imuniziranih takvom vakcinom daju prosječno više od 2 antitoksične jedinice, koliko se traži od dobre vakcine toga tipa.

Ispitivali smo i mogućnost primjene iste metode za purifikaciju tetaničkog toksoida. Rezultati tog ispitivanja za sada zadovoljavaju. Tako priređena vakcina sadržavala je 10 Lf/ml. sa 800—1000 Lf/mg. PN i titar u serumu zamoraca od 4—8 jedinica u ml.

Sada još traju ispitivanja stabiliteta kao i antigene vrijednosti tih vakcina na terenu.

CENTRALNI HIGIJENSKI ZAVOD
ODSJEK ZA DIFTERIJU
ZAGREB

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