Zag. Vet. J. (ISSN. 1110-1458) Vol. 33, No. 1 (2005) PP.204-210

Trials for preparation of *Clostridium tetani* toxoids by using different chemicals

Kamal El-Deen Hussein*; Magda, A.Kalad and Ibraheim M.A. Soliman****
*Bact. Serum and Antigens Dept. Vet. Serum and Vaccine Res. Instit,
** EquineViral Dis. Vaccine Res. Dept. Vet. Serum and Vaccine Res. Instit .

ABSTRACT

The use of different chemical in activators for modification of *Cl.tetani* toxin was studied. Formalin is still used until now as a good toxoiding agent. The attention is directed to the study of other detoxifying agents due to some objections of using formalin it causes destruction of some of the toxin antigens, if added in high doses, formalin is an excellent cross linking agent with the impurities in the medium and it needs 3-4 weeks incubation to complete toxoiding. Different concentrations of Binary ethylenimine (BEI) were tried in this study.

The toxin treated BEI was compared with the formalized toxin by flocculation test, challenge test, ELISA technique SDS page and irreversibility. The obtained results revealed that the concentration 0.08 BEI was the best concentration that gave complete toxoiding in a short period (3 days) with no reversibility, and kept the antigencity of the toxoid.

INTRODUCTION

Tetanus is an acute, often fatal bacterial disease, in which the clinical manifestations are due to the massive release of potent toxins. Therefore, the disease can be prevented by the presence of toxin-neutralizing antibodies, which can be introduced through active immunization *(1)*.

An effective method to detoxify the tetanus-toxin by formaldehyde treatment, was described by Hopkins in England (2) and Ramon in France (3).

The choice of the in activator can be made on the basis of concentration of the compound, the rate of inactivation, the availability of the compound and the inactivation time *(4)*.

The high concentration of formalin hastened the toxoids in a shortened period required for its repining but there was an increased destruction of the antigens *(5,6)*.

Abdel Fattah (7) used 2 concentrations of BEI for the inactivation of *Cl. Toxin* .

The present study was planned to compare the inactivation power of binary ethyleneimine (BEI) at different concentrations and formalin (currently used as an in activator) in the inactivation of *Clostridium tetani* toxin (2) . Moreover, the evaluation of the immunogenic capacity of the produced toxoids was considered.

MATERIAL AND METHODS

Strains: Harvard strain 49205 of Cl.tetani was used. It was obtained as lyophilized ampoule from the Egyptian Organization for Biological Products and Vaccines. VACSERA , Agoza, Cairo, Egypt.

Standard toxin and antiserum: They were obtained from the Division of Biological Standards, N.I.H., Bethesda, Maryland, USA.

Inactivator:

1- Formalin: It was used as 37% formaldehyde solution: It was obtained from the BDH LTD, England.

2- Binary ethyleneimine (BEI): This was formed through cyclization of 1 M 2-bromoethyamine hydrobromide in previously warmed 2 N sodium hydroxide (NaOH) in water bath at 37°C. The solution was immediately used as inactivator *(8)*.

Preservative: Merthiolate was prepared as 10% solution and added at a final concentration of 1:10000.

Swiss Mice: 150 mice weighing 15-20 g each were used for the determination of the minimal lethal dose (MLD) of the toxin, safety test, residual toxicity, challenge test, and irreversibility of the toxoids.

Materials for SDS-page:
Acylamide (Merck-Schuchardt)

Bis-acylamide (Sigma)
TEMED
1.5 Tris-Hcl buffer (pH8.8)
Laury sulphate (sodium dodecyl sulphate. Sigma)
Glycine (electrophoresis grade)
Ammonium presulphate (Sigma)
Glycerol (Honil Limited)
Beta mercaptoethanol (Park scientific)
Bromophenol blue (Sigma)
Comassie Brilliant blue R250
Standard protein, broad range 214-6.8 kd. (Bio-Rad).

Preparation of tetanus toxin and formalinized toxoids: The toxin was prepared from according to *(9)*. Tetanus toxoid was prepared from the highly toxigenic Harvard stain of *Clostridium tetani* which was grown in a semi synthetic medium for about one week, until the bacteria were lysed and released tetanus toxin in supernatant zone. The filtrate was detoxified by adding formaldehyde to a final concentration of 0.5%. The pH was adjusted to 7.6 with incubation at 37 °C for 3-4 weeks.

Preparation of tetanus toxoid by using Binary ethylenimine (BEI): The toxoid was prepared according to *(8)* where concentrations of 0.008 , 0.01, 0.03 0.06 and 0.08 M of BEI were added to the toxin samples. The last concentration (0.008 m) was added to the toxin in one step (on shot) , or divided into two halves and added in two steps (two shots) with 24 hrs. interval.

The 2 steps treated toxin with 0.08 M BEI (2 shots) proved to be the toxoid of choice. It was compared with the formalinized toxin by flocculation test *(10)*, challenge test *(11)* , ELISA technique *(12)* SDS – page *(13)* and irreversibility test *(14)*.

RESULTS AND DISCUSSION

The tetanus toxin is a potent neurotoxin that is synthesized intracellularly by *Clostridium tetani* as a single polypeptide chain of 150.500 Da. After cell lysis, the toxin is released in the medium and cleaved by endogenous protease to give NH_2-terminal light chain of 52.300 Da. and COOH-terminal heavy chain of 98.300 Da. The light and heavy chains are held together by a disulphide bridge *(15)*. During the detoxification process, formalin reacts with the toxin-molecules, peptones and other proteins present in the medium *(16,17, 18)*. When the formalin is added to the culture, the supernatant, containing the toxin molecules, is surrounded by a molar excess of peptones. The main reaction that occurs is a cross-linkage between the peptones and the toxin molecules. These peptides are unnecessary antigenic determinants of bovine origin that are covalently linked to the toxoid and they might be responsible for some of the side effects associated with tetanus vaccination.

The attentions was directed to show the detoxifying effect of an Aziridin derivative (Binary ethyleneimine (BEI) with different concentrations as a model which acts on the sulfadryl groups in proteins *(19, 20)*.

The inactivation rates of toxin, using BEI, are demonstrated in table (1). Complete toxoiding, using BEI concentrations of 0.008, 0.01, 0.03, 0.06 and 0.08 M (one shot) and 0.08 M (2 shots), occurred at 19 , 14, 7, 6, 4, and 3 days respectively post-treatment.

Also, the MLD of the tetanus toxins was determined only for the concentration of 0.03, 0.06, 0.08 (2 shots) (Fig. 1). The MLD of the control toxin was 150000 in mice and decreased slowly at 37°C incubation. The MLD of the toxin, treated with 0.03 M BEI, was decreased gradually till it was completely inactivated after 14 day incubation. Moreover the MLD of toxin, treated with 0.06 M BEI decreased fast till complete inactivation after 7 days incubation. While the MLD of toxin treated with 0.08 M BEI (added to the toxin in one shot), decreased faster till complete inactivation after 4 days incubation. Moreover, the toxin treated with 0.08 M BEI (added to the toxin in two shots with 24 hours intervals) showed a sudden decrease in the MLD till complete inactivation after 3 day incubation.

These results donot agree with *(7)* who used BEI for the inactivation of cl. Toxins at concentrations of 0.1% and 0.2% . He revealed that complete toxoiding took place after 20 and 15 day incubation respectively.

The toxin, treated with 0.08 M BEI, was compared with the formalinized toxin by flocculation test, challenge test, ELISA and SDS-page.

The flocculation test showed that the BEI toxoid gave 45 limit of flocculation (Lf) with kf (15 min.), while the formalinized toxoid gave 40 Lf with kf (18 min.) which indicated that BEI toxoid gave higher Lf than formalinized one.

In challenge test BEI toxoid gave relatively higher protective power than the formalinized toxoid as shown in Table (2).

The results of ELISA technique were applied according to Fig. (2) revealed that the toxin inactivated with BEI gave higher absorbance value (ranging from 2.3 – 0.49) than the formalinized toxoid (ranging from 1.7 – 0.7).

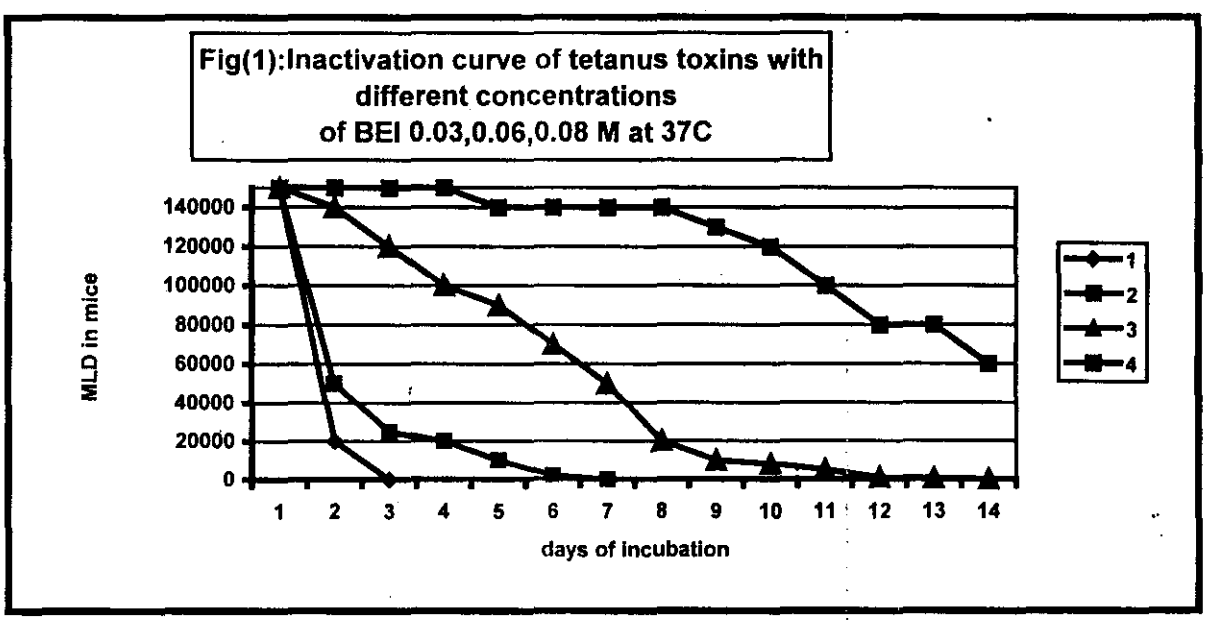
The SDS-page was applied to compare between the effect of BEI and formalin on *Cl. Tetani*-toxin. This was based on the action of both inactivators, loss of sulphydryl groups by BEI may result in so small molecules enough to pass the gel. On the contrary, the bridging action of formalin should keep the protein size visible at M W 98, 89, 101.4 Kd. (lanes 1 and 2).

Although the number of the protein bands was equal (9 bands) in the single and double-shot BEI treatment, lanes 3 and 4 there was still difference between sudden and intervalled BEI inactivator where R_{11} and R_{13} were visible respectively.

As the challenge revealed; nearly a similar response was obtained in both the BEI and formalin toxoids. It might be possible that the proteins of M.W. 98, 89, 101.4 Kd. played no major role in the protection as shown in fig (3) and table (3).

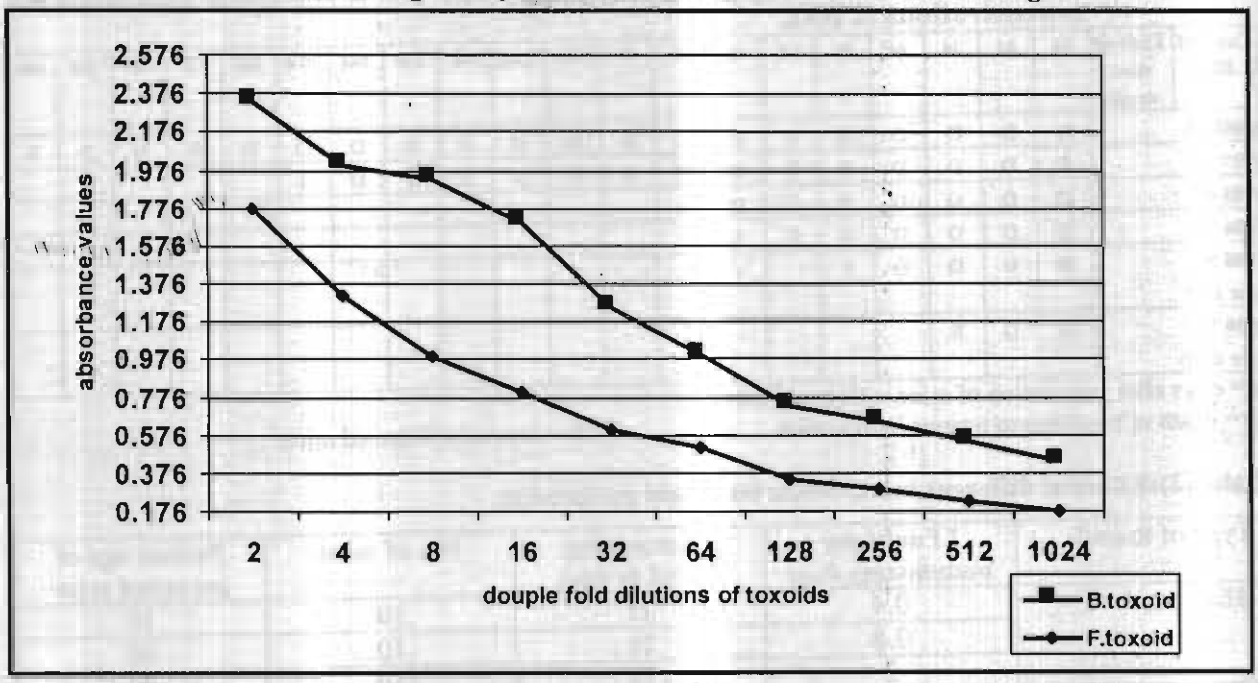
The produced BEI-toxoid was tested for irreversibility. No sample produced any sign of a toxic reaction, attributable to tetanus toxin which indicated that the BEI produced a stable toxoid .

In conclusion, for complete toxoiding, the toxin treated with formalin need 3-4 weeks incubation, while that treated with BEI took only 3 days. This gave the BEI toxoiding an advantage over the formalin, besides keeping the antigencity of the toxoid and its irreversibility.



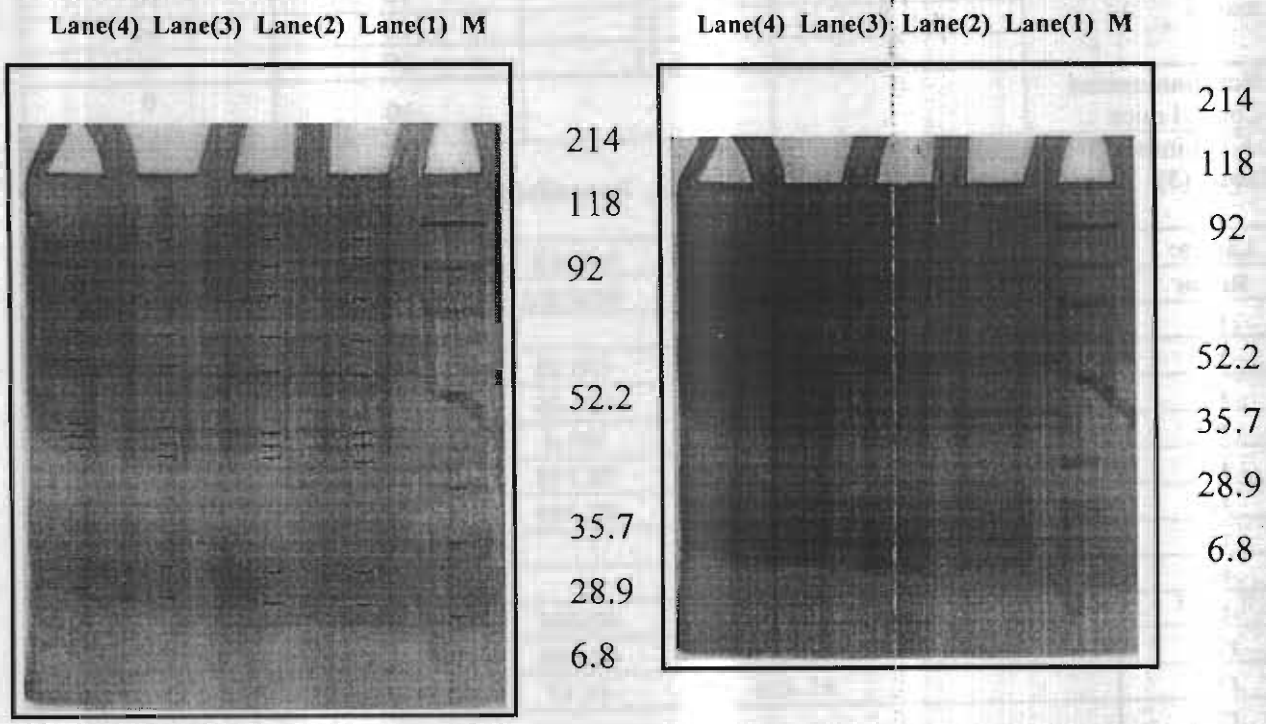
1=toxin treated with 0.08 M BEI
2= toxin treated with 0.06 M BEI
3= toxin treated with 0.03 M BEI
4= toxin without BEI

Fig (2): Estimation of the antigenicity of BEI and formalinized toxoids using ELISA.



F. toxoid = formalinized toxoid

B. toxoid = binary ethyleneimen toxoid (BEI)



Fig(3):SDS-page electrophoresis to compare between formalin and BEI toxoids

A. Scanning

B. SDS. Stained with comesis

M=marker

Lane (1) =toxin.

Lane, (2) =formalainized toxoid.

Lane (3) =BEI 0.08 toxoid one-shot

Lane (4) = BEI0.08 toxoid two shots.

Table (1): Residual toxicity of several aliquots of Cl.tetani toxins modefied with different concentrations of BEI.

Conc. of BEI / Time of mice death	1d	2d	3d	4d	5d	6d	7d	8d	9d	10d	11d	12d	13d	14d	15 d	16d	17d	18d	19d	20d
0.008 M	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	S
0.01 M	D	D	D	D	D	D	D	D	D	D	D	D	D	D	S					
0.03 M	D	D	D	D	D	D	D	S												
0.06 M	D	D	D	D	D	D	S													
0.08 M one shot	D	D	D	D	S															
0.08 M two shots	D	D	S																	

d = days after inoculation of mice with modified toxin .

D = death of mice due to tetanus intoxication. S = survive of injected mice.

Table(2):Effect of different inactivators on toxoid antigencity.

Type of toxoid	Lf units per immunizing dose	Immunizing period in days	No. of mice	Percent age of protected mice
BEI toxoid	0.4	21	10	10
	0.8	21	10	30
	2	21	10	80
Formalinized toxoid	0.4	21	10	10
	0.8	21	10	40
	2	21	10	90
Non-immunized Control mice	0	-	10	0

L.F.:- Limit of floculation

Table (3): Comparison between Cl.tetani toxin, formalinized and BEI toxoids by SDS-page electrophoresis.

Lanes: / Rows	Lane 4 (mol.w.)	Lane 3 (mol.w.)	Lane 2 (mol.w.)	Lane 1 (mol.w.)	Marker (mol.w.)
r1					214
r2	176.05	179.4	178.28	181.63	
r3	123.58	120.23	121.35	139.21	118
r4			101.4	98.892	92
r5	82.419	80.453	79.716	77.751	
r6	68.66	65.712	62.764	61.536	
r7					52.2
r8	47.268	46.551			
r9	45.116		45.564	45.564	
r10	42.874	43.681	43.233	43.412	
r11		41.439	41.17	40.363	
r12					35.7
r13	24.359				28.9
r14		21.937	20.272	20.121	
r15	16.034	12.855			
r16			11.341	10.584	6.8

Lane1= Cl.tetani toxin Lane2= formalinized toxoid

Lane3= 0.08 BEI toxoid (BEI added in one shot)

Lane4== 0.08 BEI toxoid (BEI added in two shot with 24 h.intervals)

REFERENCES

1-Radositis D. M, Blood D. C, Arundel J. H and Gaydvm E. C (2000): A textbook of Vet. Medicine 9th Ed. English Language Book Society Ballerie Tindel London, England.

*2-Glenny AT and Hopkins BE. (1923):*Diphtheria toxoid as an immunizing agent. Br.J.Exp.Pathol. 4:283.

3-Ramon G.(1924): Sur la toxine et sur l'anatoxine diphtheriques. Ann Inst.Pasteur.38:1.

4- Hassanien, M. M. (1992): Prilimenary findings for an inactivated A. H. S. Vaccine using Binary ethyleneimine. Reviewe Elev, Med, Vet., Pags Trop., 45 (3-4) : 231-234.

5- Nedjalkov, S. and Peichve, B. (1974): Studies on the toxin of Cl.perferingens type C. Vet.Nauki-Bukgaria, 11, No2:73-78.

6- WHO (1978): Manual for the Production and Control of Vaccines." Tetanus toxoid" BIG/VNDP/77.2 Rev.1.

7- Abdel. Fattah, A. M. (1996): Compartive study on the immune response of sheep and goat against Cl. vaccines. Ph. D. Thesis, Faculty of Vet.Med. Cairo Univ.

8- Girard, House, Peter, E.Mikiciok and Mary Lou Beringer (1990): Laboratory diagnosis of AHS. J.Vet.Diag.Invest. 2:44-50.

9- Mueller, J.H. and Miller, P.A. (1954): Variable factors influencing the production of tetanus toxin production J.Bact.67: 271-277.

10-Norris, J.R. and Ribbons, D.W. (1971): Methods in Microbiology. Vol. III Academic press, London and New York.

11- Soares,H.R. ; Nunes,R.L. ; Bprado,S.M. ; Higashi,H.G. and Heneine,I.F.(1994): An effective toxoid from tetanus toxoid prepared by stepwise iodination of purified toxin. Biologicals, 22(1):29-33.

12-Carol, House, Peter, E. Mikiciok and Mary Lou Beringer (1990): Laboratory diagnosis of AHS J. Vet. Diag. Invest. 2: 44-50.

13- Laemmli, V.K., (1970): Nature 227:680.

14-European Pharmacopoeia (1997): Tetanus vaccine.1616.

15-Bizzini B. (1984): Tetanus. Germanier R, ed. Bacterial Vaccines. New York: Academic Press, 37.

16- Blass J. (1964): Etat actuel de nos connaissances sur le mecanisme de la detoxification par le formol. Biol. Med, 53:202.

17- Ray R.; Dotta KK, Mukherjee AK, Anand BR, Rai Chowdhuri AN. (1983): Hospital based surveillance of faucial diphtheria in and around Dethi. Indian J.Med.Res., 78:776.

18- Pappenheimer AM Jr. (1984): Diphtheria. In: Germanier R,ed. Bacterial Vaccines. New York: Academic Press,.

19- Raftery, M.A., and Cole R.D. (1963): Tryptic cleavage at cysteinyl peptides bonds. Biochem.Biophys.Res.Commun.10:467-472.

20-Bahnemann, H.G., Ague Due Melio,P., Abracon,D. and Gomes, I. (1974): Immunogenicity in cattle of FMD vaccines inactivated with Binary ethyleneimine. Bull.Off.Int.Epiz. 81(11-12):1335-1343.

الملخص العربى

محاولات لتحضير توكسيد الكلوستريديوم تيتاناى بإستخدام مواد كيمائية مختلفة

د.كمال الدين حسين*، د.ماجدة أنيس قلد** أ.د.إبراهيم محمد أحمد سليمان**

*قسم بحوث الأمصال و الأنتيجينات البكتيرية ، ** قسم بحوث لقاحات الفصيلة الخيلية

استهدفت هذة الدراسة تطوير الكلوستيريدم تيتاناى باستخدام مواد كيميائية مختلفة وبالرغم من استمرارية استخدام الفورمالين كمثبط فإن له بعض العيوب التى تؤثر على إنتاج توكسيد نقى منها تدمير بعض الإنتجينات الموجودة فى التوكسين عند إضافته بكميات كبيرة وعمل روابط قوية بين جزئ التوكسين والبروتينات الموجودة فى
المستنبتات الغذائية بالإضافة إلى أنه يحتاج إلى ٣ – ٤ أسابيع حتى يتحول التوكسين إلى توكسيد.

لذلك تحت دراسة تأثير البيناري إثيلين امين كمادة مثبطة بتركيزات مختلفة (٠,٠٠٨ ، ٠,٠١ ، ٠,٠٣ ، ٠,٠٦، ٠,٠٨) وعمل دراسة مقارنة لكل من التوكسيد المحضر باستخدام الفورمالين والتوكسيد المحضر بالبيناري إثيلين أمين بإستخدام اختبارات : التندفة (flocculation test) ، التحدى فى الفئران والاليزا واختبار التحليل الكهربى للبروتينات وأسفرت النتائج بأن التوكسين المعامل بمادة البيناري بتركيز ٠,٠٨ هو أفضل من التوكسين بالمعامل بالفورمالين حيث أنه يحوله إلى توكسيد فى مدة قصيرة (٣ أيام) دون التأثير على قوته المناعية.

والخلاصة أنه يمكن الاعتماد على مادة البيناري إثيلين أمين فى تحضير التوكسيد لميكروب التيتانوس بفاعلية وأمان مما يشجع على إمكانية استخدامه مستقبلا فى إنتاج لقاح لهذا المرض.