BBA 3985

SOME STUDIES ON THE CHEMICAL MODIFICATION OF ε-TOXIN OF *CLOSTRIDIUM PERFRINGENS* TYPE D

A. F. S. A. HABEEB*

Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario (Canada)

(Received October 29th, 1962)

SUMMARY

Chemical modification of ε-toxin of *Clostridium perfringens* type D by acetylation and guanidination has been carried out. With both reagents when 50 % of the free amino groups were reacted the toxicity dropped to 7 %. Further modification of the free amino groups by guanidination reduced the toxicity to 0.6 % and with acetylation the toxicity was completely abolished. The modified toxins showed loss in their power to combine with the antiserum. Modification of ε-toxin with formalin resulted in complete loss of both toxicity and combining power. The modified toxins exhibited antigenic properties.

INTRODUCTION

Chemical modification of biologically active proteins has proved useful in revealing the chemical nature of the group(s) responsible for their activity. Although bacterial toxins form an important group of biologically active proteins, very little work has been done on their chemical modification. HEWITT[1], GOLDIE[2,3] and PAPPENHEIMER[4] inactivated diphtheria toxin with formalin and ketene and observed a parallelism between the disappearance of the free amino groups and loss of toxicity. The reagents used lacked the specificity necessary for defined chemical modifications[5–7]. To obtain additional insight into this process chemical modification of the amino groups of ε-toxin of *C. perfringens* type D has been investigated in this laboratory by acetylation and guanidination using acetic anhydride[7] and 1-guanyl-3,5-dimethylpyrazole nitrate[8] which are known for their specificity; formalin was also included since it is used extensively for preparation of toxoids. The effect of the chemical modification on toxicity, combining power and antigenicity is reported.

MATERIALS AND METHODS

GDMP was synthesized[9]. Trypsin twice crystallized and soybean trypsin inhibitor were obtained from Nutritional Biochemical Corporation. Standard toxin, antitoxin and

Abbreviations: GDMP, 1-guanyl-3,5-dimethylpyrazole nitrate; MLD, minimum lethal dose; BSA, bovine serum albumin.

* Present address; Roswell Park Memorial Institute, Buffalo, N.Y. (U.S.A.).

bacterin were obtained from U.S. Department of Agriculture, Agricultural Research Service.

Prototoxin

C. perfringens type D produces a prototoxin[10] which is activated by trypsin to ε-toxin. The prototoxin was obtained by growing *C. perfringens* type D in a modified liquid medium[11] consisting of proteose peptone, NZ case, lactalbumin hydrolysate, yeast extract, dextrin, phosphate and cysteine at a controlled pH of 7. After 24 h the cells were centrifuged and to each liter of supernatant 600 g ammonium sulphate were added and the solution was held overnight at 4°. The crude prototoxin that floated was collected, washed with saturated $(NH_4)_2SO_4$ solution, dissolved in water and dialyzed exhaustively against distilled water and freeze-dried.

Toxin

The freeze-dried prototoxin was dissolved in 1/15 M phosphate buffer (pH 7.5) and dialyzed against buffer. The protein concentration was determined by N analysis (25 mg/ml). To 100 mg of prototoxin was added 1 mg of crystalline trypsin and the solution was incubated at 37° for 45 min. Then soybean trypsin inhibitor was added (1.6 mg per mg trypsin) and the mixture was incubated at 37° for 20 min. Using the spot plate method with *p*-toluenesulfonyl arginine methyl ester[11] as substrate no trypsin was detected 1 min after adding the trypsin inhibitor. Inhibiting the trypsin activity was necessary since it was found that the toxin was destroyed on standing with trypsin and this was in agreement with results of TURNER AND RODWELL[13]. The activated toxin solution was then dialyzed against water to remove dialyzable nitrogen.

Diluent used for diluting samples for toxicity and combining power determination consisted of 1 % peptone (Difco) in 0.25 % NaCl solution adjusted to pH 6.8–7.2.

Toxicity

White mice 18–20 g were injected intravenously with 0.5 ml solution and deaths occurring within 24 h were recorded. Toxicity was expressed as MLD/mg toxin.

Combining power

This test was carried out as described by U.S. Department of Agriculture, Agricultural Research Service. The prototoxin or modified toxin was incubated with standard antitoxin for 1 h at room temperature, a toxin indicator dose was added and the mixture incubated for an additional hour. Then 0.5 ml of the mixture was injected into mice and mortality observed up to 48 h.

Determination of antitoxin

Antitoxin titres in sera were determined by comparing the toxin-neutralizing ability of the antisera with that of the standard reference antitoxin. To do this 1 ml of serum or suitable dilution of serum was mixed with 1 ml of toxin containing 5 L^+ doses and incubated at room temperature for 1 h. Then 0.4 ml was injected intravenously in each of 2 mice and deaths occurring within 48 h were recorded. Standard antiserum containing 5 and 4 antitoxin units per ml was also used.

Free formaldehyde was determined by the method of NASH[14]. Protein concen-

tration was determined from Nessler N analysis by using a factor of 6.25. Determination of free amino groups was carried out by ninhydrin analysis[15] with some modification. To 1 ml containing the protein sample was added 0.5 ml 10 % pyridine solution and 0.5 ml 2 % ninhydrin. The solution was heated for 20 min in a boiling-water bath, cooled, and diluted to 10 ml with water. A calibration curve for prototoxin was constructed at 0.15–1.6 mg protein/ml.

RESULTS

Control on conditions of chemical modification

It was necessary to establish the stability of the toxin under conditions of temperature and pH existing under the conditions of acetylation, guanidination and formolation.

Control on acetylation: To 4 ml of toxin containing 2.5 mg protein/ml was added 0.6 g sodium acetate. Then 0.1 ml acetic acid was added gradually while keeping the pH at 7 with 1 N NaOH. The solution was dialyzed against water overnight and then neutralized to pH 7. The protein nitrogen and toxicity were determined.

Control on guanidination: To 1 ml of toxin containing 5 mg protein/ml was added 1 ml of borate buffer (pH 9.5) and merthiolate to a concentration of 1:10 000. The solution was held at room temperature for 24 h, dialyzed against water and assayed for toxin.

Control on formalin treatment: Aliquots of 1 ml of toxin containing about 2 mg protein/ml and 1/10000 merthiolate were kept at 37° for 3, 6 and 16 days, after which toxicity was determined and expressed as MLD/ml.

Table I reports on the stability of the toxin subjected to the treatments described above.

Treatment of ε-toxin with formalin

Three aliquots of 15 ml of toxin solution containing about 2 mg protein/ml were treated with formalin at 0.1, 0.3 and 0.5 % concentration. At intervals 1-ml aliquots were removed for determination of free formaldehyde and toxicity. Table II shows the effect of formalin treatment on toxicity and combining power of the toxin.

Reaction of acetic anhydride with ε-toxin

Acetic anhydride was added to 4 ml toxin solution (3 mg protein/ml) containing

TABLE I

STABILITY OF ε-TOXIN

	MLD/mg	*MLD/ml*
Toxin dialysed	17 500	
Toxin treated with sodium acetate	16 500	
Toxin kept at pH 9.5	17 500	
Toxin solution (original)		26 000
Toxin solution at 37° for 3 days		26 000
Toxin solution at 37° for 6 days		26 000
Toxin solution at 37° for 16 days		22 000

sodium acetate at 1/5 saturation. Where low degrees of acetylation were required acetic anhydride was diluted in dioxane[16] to obtain 2, 4 and 10 % of the acetic anhydride. The reaction was performed at room temperature for 15 min. The pH was found to drop from 8 to 7.2, 6.8, 6.7 and 6.3 for 0.0008, 0.002, 0.004 and 0.008 ml acetic anhydride respectively. When greater amounts of acetic anhydride was used,

TABLE II

THE EFFECT OF FORMALIN ON ε-TOXIN

Time after formalin treatment (days)	*Formaldehyde (p.p.m.)*			*Toxicity (MLD/ml)*			*Combining power (units/ml)*		
	0.1 %	*0.3 %*	*0.5 %*	*0.1 %*	*0.3 %*	*0.5 %*	*0.1 %*	*0.3 %*	*0.5 %*
0	400	1080	2110	26 000	26 000	26 000	3000	3000	3000
1	374	1026	1950	4000	1000	20	—	< 100	< 100
5	—	—	1740	—	—	0	—	—	< 10
7	345	1006	—	500	2	—	—	—	—
9	—	—	—	—	0	—	—	< 10	—
14	327	—	—	200	—	—	500	—	—
22	330	—	—	100	—	—	—	—	—
28	—	—	—	50	—	—	—	—	—

the pH was maintained at 7 by addition of 1 N NaOH. The sample was then dialyzed overnight against distilled water. Aliquots were used for determination of protein N and percentage of free amino groups. Toxicity of the preparation was determined and expressed as MLD/mg protein; the percentage of toxicity was calculated using non-modified toxin as 100 %. Fig. 1 shows the residual free amino groups as a function of quantity of acetic anhydride. Maximum acetylation was obtained by using 0.01 ml acetic anhydride and was not influenced by increasing this reagent 20-fold.

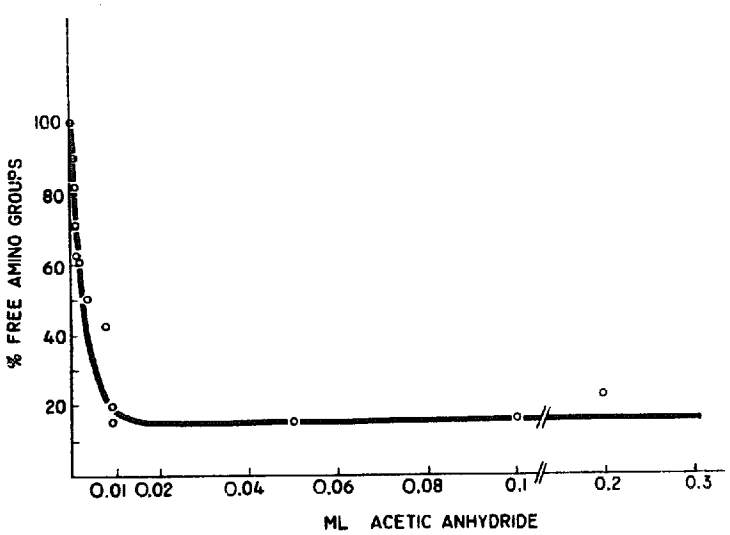

Fig. 1. Reaction of acetic anhydride with ε-toxin.

Reaction of GDMP with ε toxin

GDMP was dissolved in water and the pH adjusted to 9.5 with 1 N NaOH. Toxin was added to give 10 mg protein/ml in 0.3 M GDMP. The reaction was performed at room temperature and pH 9.5. 1-ml samples were taken at intervals, diluted and

neutralized to stop the reaction, dialyzed overnight and again neutralized. Similar reactions were also performed in a 0.5 M GDMP solution. The percentages of free amino groups, protein concentration and toxicity were determined and the percentage of toxicity calculated. Fig. 2 shows the percentage of residual free amino groups as

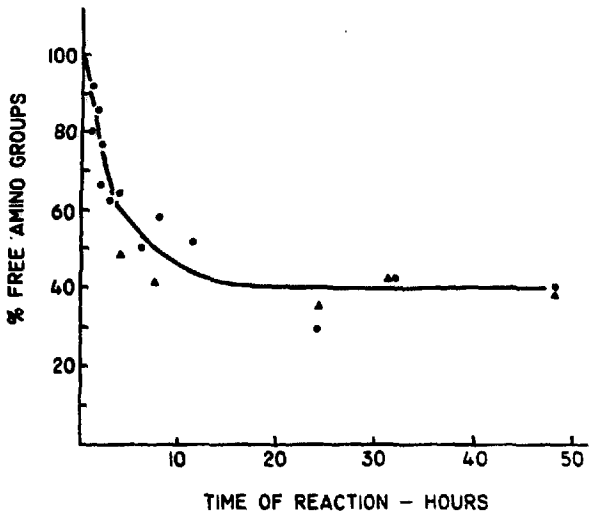

Fig. 2

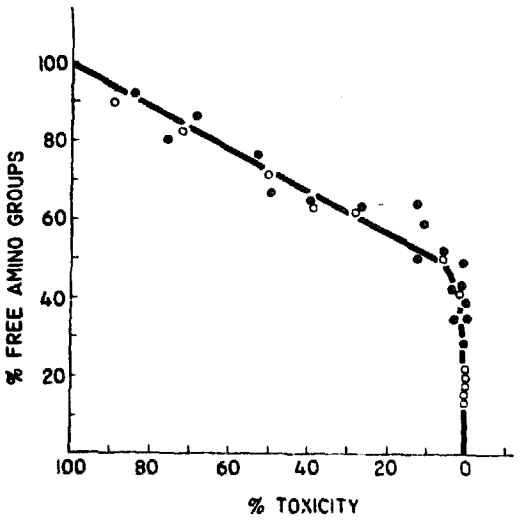

Fig. 3

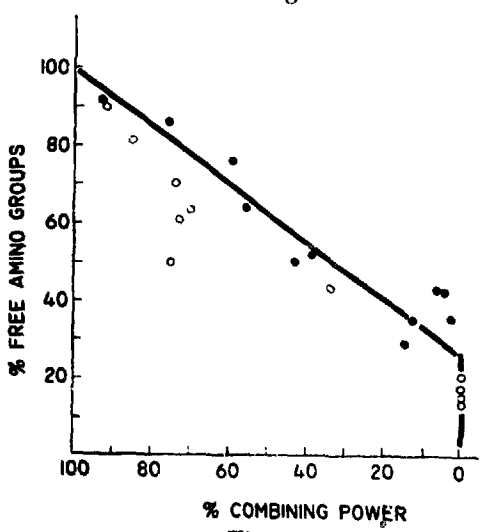

Fig. 4

Fig. 2. Reaction of GDMP with ε toxin. ●—●, 0.3 M GDMP; △—△, 0.5 M GDMP.

Fig. 3. Effect of chemical modification of amino groups on toxicity of ε-toxin. ○—○, acetylation; ●—●, guanidination.

Fig. 4. Effect of chemical modification of amino groups on combining power of ε-toxin. ○—○, acetylation; ●—●, guanidination.

function of reaction time. About 40% of the amino groups remained free with maximum guanidination being obtained in 24 h. Neither increasing the reaction time to 48 h nor increasing the concentration of GDMP to 0.5 M had any effect on guanidination.

Effect of acetylation and guanidination on toxicity

Toxicity decreased with decrease in free amino groups and reached about 7% when 50% of the free amino groups were either acetylated or guanidinated (Fig. 3). With guanidination complete loss of toxicity was never attained, the minimum value being 0.6% toxicity with 39% free amino groups. Complete loss of toxicity was obtained by acetylating 80% of the amino groups.

Effect of chemical modification on combining power

Toxin treated with 0.3 and 0.5 % formalin lost its power to combine with antiserum while that treated with 0.1 % retained about 1/6 of its combining power. Acetylated and guanidinated toxin showed loss in their combining power with decrease in free amino groups (Fig. 4).

Antigenity of the modified toxin

Ability to elicit antibodies in guinea-pigs was studied using the following antigens: (a) toxin guanidinated with 0.3 M GDMP at pH 9.5 for 24 h, (b) acetylated with acetic anhydride in 1/5 saturated sodium acetate (0.03 ml/24 mg protein) and (c) treated with 0.3 % formalin at 37° for 7 days. The residual free amino groups, toxicity and combining power were determined. Table III gives the properties of the modified toxins.

TABLE III

PROPERTIES OF MODIFIED TOXINS

Preparation	*Free amino groups (%)*	*Combining power (units/mg)*	*Residual toxicity (MLD/mg)*
Guanidinated toxin	37	100–200	200
Acetylated toxin	22	50–100	0
Formalin-treated toxin	Not determined	0–10	< 20

TABLE IV

THE ANTITOXIN LEVEL IN GUINEA-PIGS AFTER INJECTION WITH MODIFIED TOXIN

Before immunization all guinea-pigs showed a level of < 0.5 A.U.* in the three toxins tested

	Acetyl toxin (A.U.)	*Guanyl toxin (A.U.)*	*Formalin-treated toxin (A.U.)*
3 weeks after 1st injection	< 4 in all	16, 16, 32, 180–256	< 4, 5–8, 16, 10–16, 20–32
3 weeks after 2nd injection	< 4, < 4, < 4, 4	80–96, 160–192, 80–96, 400	160–192, 320, 320, 1600

* Abbreviation: A.U., antitoxin unit.

To prepare an antigen for injection, the modified toxin was mixed and homogenized with an equal volume of a mixture of 9 parts Bayol F and 1 part Arlacel A. This gave 1 mg protein/ml for acetyl and guanyl toxin and 2 mg/ml for formalin-treated toxin. 1 ml of the vaccine was injected intramuscularly in each of 5 guinea-pigs. With guanyl toxin it was necessary to give the 1 ml in 3 doses over one week owing to the residual toxicity. A second dose of 1 ml was given 3 weeks after the first injection. The guinea-pigs were bled 3 weeks after both the first and second injection. The sera were separated and titrated for antibody level. Results are given in Table IV.

DISCUSSION

In chemical modification of biologically active proteins it is essential to determine that the loss in activity is not due to non-specific inactivation and therefore controls

on the conditions of the reaction have to be included. Table I showed that ε-toxin was stable under the conditions of acetylation and guanidination, although a slight decrease in toxicity took place during incubation at 37° for 16 days.

Chemical modification may result in configurational changes of the protein molecules as a result of substitution, therefore two reagents were used which are specific for the amino groups. Acetic anhydride[17] and GDMP[8,18] replaced the positively charged NH_3^+ group by an uncharged group and a positively charged $NH{-}C(NH_2)_2^+$ group respectively. Formalin was also studied since it is popularly employed for the preparation of toxoids.

The results in Table II showed that the time necessary for toxoiding varied with the concentration of formalin and that although there was an excess of formaldehyde, toxoiding was very slow and incomplete with 0.1 % formalin even after 4 weeks. There was some fixation of formaldehyde as shown by the decrease in free formaldehyde which is to be expected since formaldehyde is known to react with the free amino groups[5,6]. These results are not in agreement with those of FULTHROPE AND THOMSON[19] who reported no significant fixation of formaldehyde during toxoiding of tetanus toxin. The loss in combining power as a result of formalin treatment was significant and was almost complete with 0.3 and 0.5 % formalin and was similar to results reported by SCHUCHARDT *et al.*[20,21] for ε-toxin of *C. perfringens* type D. Even with 0.1 % formalin there was about 83 % loss of combining power.

It was found possible to acetylate 85 % of the free amino groups of ε-toxin by reacting 0.01 ml acetic anhydride and 12 mg toxin at room temperature for 15 min (0.83 ml acetic anhydride/g protein). On the other hand guanidination using GDMP only resulted in reaction of about 60 % of the free amino groups after 24 h at room temperature. The residual free amino group may be either resistant lysine or free N-terminal amino groups or both, since GDMP has been shown to react incompletely with N-terminal amino groups[8].

The decrease in free amino groups after acetylation or guanidination caused a loss of toxicity which was complete with the former. The loss of toxicity was similar in both cases although the net charge of both preparations would be expected to be different due to the substitution of positively charged ammonium groups in one case by uncharged acetamido groups and in the other case by a positively charged guanidino group. These results may show that acetylation or guanidination of the toxin may have little or similar effect on the molecular configuration of the protein molecules. If as in the case of β-lactoglobulin where acetylated[22] and guanidinated[8] β-lactoglobulin had similar electrophoretic mobility then similar configurational changes in the modified toxin would take place. On the other hand, the site responsible for toxicity may be very stable towards the changes in the net charge due to acetylation or guanidination. Physico-chemical studies on the pure unmodified and modified toxin would be necessary for clarification of this point.

The chemical modification of the amino groups of the toxin resulted in a decrease in the power of modified toxin to combine with antiserum (Fig. 4) and complete loss is obtained with 20 % free amino groups. The points for acetylated toxin showed greater scatter than with the guanidinated derivative and may represent the tendency of some *N*-acetyl groups of the acetylated toxin to hydrolyze readily. Several of the acetylated derivatives caused death in mice after 24 h. This was most probably due to hydrolysis of the acetyl group *in vivo* because ε-toxin usually kills mice in less than

24 h. Hydrolyzable *N*-acetyl groups have been reported in acetylated porcine pancreatic amylase[23].

Since chemical modification of 50 % of the free amino groups of ε-toxin caused it to lose about 93 % of its toxicity and 65 % of its power to combine with the antiserum, it is likely that the amino groups responsible for the toxicity are different from those necessary for the combining power. The former may represent groups more susceptible to modification than the latter due to their location in the protein molecule.

The results (Fig. 4) show that ε-toxin behaves differently to protein antigens on chemical modification of the amino groups with respect to its power to combine with antiserum. MARRACK AND ORLANS[24] found that acetylated derivatives of bovine serum albumin, ovalbumin and lysozyme were found to retain 67, 75 and 86 % respectively of their power to precipitate with the respective antibody and SINGER[25] found that guanidinated bovine serum albumin retained 90 % of its capacity to precipitate anti-BSA antibody. In contrast, ε-toxin lost about 95 % of its combining power when 70 % of its amino groups were modified by either acetylation or guanidination.

This may be a general property of toxins or of toxins with similar modes of action and emphasizes the importance of the interaction of the amino groups of the toxin with the reactive sites of the homologous antibody. Since the work of GROSSBERG AND PRESSMAN[26] indicated a carboxyl group at the site of antibody against a positively charged group one may speculate that antibody directed against ε-toxin may contain a negatively charged carboxyl group at the specific site.

The results on the antigenicity of the modified toxin showed that although they lost most of their combining power yet they were antigenic. No attempt is taken at this time to compare the antigenic power of the modified toxins. Acetyl toxin produced a poor response under the conditions of the test. One cannot conclude whether the guanyl toxin is a good antigen or the response was due to the residual toxicity. Further experiments on partially modified and completely modified toxins will be necessary to establish the merits of the modified toxins as antigens.

The combining power and antigenicity appear to be two different properties of toxin or modified toxin. Amino groups are necessary for toxin to combine with homologous antibodies so that modification of the amino groups results in a loss of the combining power. On the other hand antigenicity is due to the ability of modified toxin to elicit the production of antibodies and would be affected by configurational changes in the toxin molecules. It is therefore possible to lose the combining power without effect on the antigenicity.

These results emphasize the dangers inherent in evaluating the antigenic value of a modified toxin by its power to combine with antiserum.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Dr. H. PIVNICK for his encouragement and interest in this work.

REFERENCES

1 L. F. HEWITT, *Biochem. J.*, 24 (1930) 983.
2 H. GOLDIE, *Compt. Rend. Soc. Biol.*, 126 (1937) 974.
3 H. GOLDIE, *Compt. Rend. Soc. Biol.*, 126 (1937) 977.

[4] A. M. PAPPENHEIMER, *J. Biol. Chem.*, 125 (1938) 201.
[5] H. FRAENKEL-CONRAT AND H. S. OLCOTT, *J. Am. Chem. Soc.*, 70 (1948) 2673.
[6] H. FRAENKEL-CONRAT AND D. K. MECHAN, *J. Biol. Chem.*, 177 (1949) 477.
[7] F. W. PUTNAM, in H. NEURATH AND K. BAILEY, *The Proteins*, Vol. 1B, Academic Press, New York, 1953, p. 924.
[8] A. F. S. A. HABEEB, *Can. J. Biochem. Physiol.*, 38 (1960) 493.
[9] R. A. B. BANNARD, A. A. CASSELMAN, W. F. COCKBURN AND G. M. BROWN, *Can. J. Chem.*, 36 (1958) 1541.
[10] T. J. BOSWORTH AND R. E. GLOVER, *University of Cambridge, Institute of Animal Pathology, 4th Report*, (1934–1935) p. 79.
[11] H. GORDON, M. FIOCK, A. YARNSKY AND J. T. DUFF, *J. Bacteriol.*, 74 (1957) 533.
[12] M. R. RHODES, R. M. HILL AND R. E. FEENY, *Anal. Chem.*, 29 (1957) 376.
[13] A. W. TURNER AND A. W. RODWELL, *Australian J. Exptl. Biol. Med. Sci.*, 21 (1943) 17.
[14] T. NASH, *Biochem. J.*, 55 (1953) 416.
[15] V. J. HARDING AND R. M. MACLEAN, *J. Biol. Chem.*, 24 (1916) 503.
[16] A. F. S. A. HABEEB, H. G. CASSIDY, P. STELOS, AND S. J. SINGER, *Biochim. Biophys. Acta*, 34 (1959) 439.
[17] H. FRAENKEL-CONRAT, R. S. BEAN AND H. LINEWEAVER, *J. Biol. Chem.*, 177 (1949) 385.
[18] A. F. S. A. HABEEB, *Can. J. Biochem. Physiol.*, 39 (1961) 729.
[19] A. J. FULTHROPE AND R. O. THOMSON, *Immunology*, 3 (1960) 126.
[20] L. F. SCHUCHARDT, J. MUNOZ AND W. F. VERWEY, *J. Immunol.*, 80 (1958) 237.
[21] J. MUNOZ, L. F. SCHUCHARDT, W F. VERWEY AND S. F. SHEIDY, *Proc. Ann. Meetings U.S. Livestock Sanitary Assec.*, 58 (1955–1956) p. 62.
[22] A. F. S. A. HABEEB, H. G. CASSIDY AND S. J. SINGER, *Biochim. Biophys. Acta*, 29 (1958) 587.
[23] I. RADICHEVICH, M. M. BECKER, M. EITINGTON, V. H. GETTLER, G. C. TORALBALLA AND M. L. CALDWELL, *J. Am. Chem. Soc.*, 81 (1959) 2845.
[24] J. R. MARRACK AND E. S. ORLANS, *Brit. J. Exptl. Pathol.*, 35 (1954) 389.
[25] S. J. SINGER, *Proc. Natl. Acad. Sci. U.S.*, 41 (1955) 1041.
[26] A. L. GROSSBERG AND D. PRESSMAN, *J. Am. Chem. Soc.*, 82 (1960) 5478.