Clostridium oedematiens η -Antigen, an Enzyme Decomposing Tropomyosin

By MARJORIE G. MACFARLANE

The Lister Institute of Preventive Medicine, London, S.W. 1

(Received 26 February 1955)

Oakley, Warrack & Clarke (1947) demonstrated the existence in culture filtrates of various strains of *Clostridium oedematiens* of six antigens, designated as α , β , γ , δ , ϵ and ζ . The α -antigen is produced by both type A and type B strains and is the main lethal agent in *Cl. oedematiens* toxic filtrates. Its biochemical action is unknown, but one of its physiological effects is that of a capillary poison (A. A. Miles & E. M. Miles, 1952; A. A. Miles & J. M. Elder, unpublished), which may well be due to an attack on some constituent of the capillary wall. Since the chemistry of the capillary wall is unknown, it was decided to find out if the crude toxin had any action on some known proteins, particularly those which, by association in lipoprotein structures, might themselves conceivably be concerned in some permeability phenomenon, in the hope of getting some clue to the nature of the toxic action.

It has been found that *Cl. oedematiens* type B (*gigas*) toxins contain an antigenic sulphhydryl-activated enzyme decomposing tropomyosin. Serological tests show, however, that this enzyme is not identical with the lethal α -toxin, nor with any of the other previously described antigens. It has therefore been designated specifically as *Cl. oedematiens* η -antigen, and called a tropomyosinase. The characters of this enzyme are outlined below. Throughout this paper the word 'toxin' is used to denote the crude enzyme preparation, which was in fact toxic, and the specific antigens, distinguished in this toxin by serological tests, are denoted as α -antigen, β -antigen, etc.

MATERIALS AND METHODS

Toxins and antitoxins. For examination of the biochemical properties of the enzyme, *Cl. oedematiens* toxin (type B, strain Albiston) batch Oe8 was used. This was prepared in the Serum Department of this Institute by salting out from the culture filtrate with ammonium sulphate and drying *in vacuo*. A sample was dialysed at 2° in 7% solution in cellophane at pH 7.0 against 0.15M-NaCl and the insoluble matter removed by centrifuging. The solution (20 ml.) was accurately dispensed in amounts of 0.02 and 1.0 ml. and freeze-dried, and the ampoules were filled with nitrogen and sealed. The analytical values per ml. of solution, tested after drying, were: 1.8 mg. total N; 1.4 mg. acid-soluble N; not more than 0.6 lecithinase units

(Lewis & Macfarlane, 1953); no collagenase nor any haemolysin other than the lecithinase; and by a biological test approximately 50 L₊ units against the International Standard antitoxin. This preparation, Oe8a, was arbitrarily designated as containing 150 units of tropomyosinase/ml.

For serological identification crude toxins of *Cl. oedematiens* and horse antisera prepared against them were obtained from several laboratories named below.

Tropomyosin. This was prepared from rabbit muscle substantially by the method of Bailey (1948, 1951). After the first fractionation with ammonium sulphate, the crude tropomyosin was dialysed against 0.1M-KCl, precipitated with ethanol and dried, refractionated with ammonium sulphate and again precipitated with ethanol after dialysis. At this stage a 2% (w/v) solution in water was still faintly opalescent and 0.2 ml. gave a weakly positive test for tryptophan with Ehrlich's reagent. It was found convenient, owing to the time taken to dissolve dry tropomyosin, to make up at this stage a 2% solution in 0.1M-KCl, dialyse overnight against the solvent, centrifuge clear and store the solution, now practically tryptophan-free, at -10°. The nitrogen content was determined on a sample; suitable quantities for experiment were withdrawn, diluted as desired and stored at 2° for up to a week without apparent change in viscosity.

Myosin. This was extracted from rabbit muscle according to Bailey (1942) and was used after one precipitation in 20 vol. water and resolution in 0.3M-KCl at pH 7.0 and filtering through thick (Whatman no. 3) paper. This preparation, which probably contained some actin, is designated as (acto-)myosin as suggested by Bailey (1954).

Buffers. Stock solutions of phosphate buffer, KH_2PO_4 - Na_2HPO_4 , *I* (ionic concentration) = 1.0 at pH 6.5, 7.0, 7.5 and 8.0, were diluted as required.

Activators and inhibitors. Solutions of cysteine hydrochloride and thiolacetic acid (both brought to pH 7.0 with NaOH), glutathione and sodium iodoacetate were made up just before use.

Experimental mixtures. In most of the experiments with tropomyosin mixtures were made of 1 vol. 1% protein in 0.1M-KCl and 1.5 vol. of a mixture of toxin, cysteine and phosphate buffer in quantities adjusted to give a final concentration of 0.01M cysteine and *I* = 0.1; with (acto-)myosin *I* was 0.3. The two components were placed 5 min. in a thermostat at 25° before mixing; the mixture was immediately transferred to a viscometer and readings were timed from the time of mixing.

Viscosity measurements. These were made in Ostwald viscometers, about 2.0 ml. in capacity with flow times 50-60 sec. for water at 25°, which were arranged in sets up to five with flow times within 5 sec. The relative viscosity, η_{rel} , was taken as the ratio of the flow time of the sample to that of the solvent in the same viscometer. In kinetic

experiments the time of observation was recorded as that at the start of flow, i.e. no correction was made for the change in viscosity during the flow time. For the purposes of these experiments the results were not materially affected by the errors due to this change or by use of different viscometers.

Nitrogen determinations. These were made by micro-Kjeldahl technique. For determination of acid-soluble N, a measured volume of trichloroacetic acid was added to the sample to give a final concentration of 6.7% (w/v), and the mixture filtered after 10 min.

EXPERIMENTAL

Action of Clostridium oedematiens type B toxin on tropomyosin

Preliminary experiments showed that on incubating mixtures of tropomyosin and *Cl. oedematiens* toxin Oe8a in phosphate-KCl solution in the presence of cysteine or thiolacetate the viscosity fell progressively to a limiting value, $\eta_{rel.}$ about 1.1. In the absence of toxin, the viscosity of the mixture decreased slowly but appreciably in the presence of thiolacetate, but was unaltered in the presence of cysteine; it was also unaltered in the presence of cysteine and boiled toxin, and in the presence of cysteine, toxin and homologous antitoxin. These facts indicated the presence of an antigenic SH-activated proteinase. In subsequent experiments cysteine was used as activator.

Course of decomposition. (1) *Rapid phase.* The toxin caused a rapid decrease of viscosity and then a slower formation of acid-soluble N from tropomyosin, illustrated in the following experiment:

Expt. 1. Toxin Oe8a (125 units) in 12.5 ml. phosphate buffer (pH 7.0, $I=0.1$) and 2.5 ml. 0.1M cysteine was mixed at 25° with 10 ml. 1% tropomyosin in 0.1M-KCl. A sample was placed in a viscometer and the remainder incubated in bulk at 25°, samples being removed at intervals for determination of the acid-soluble N. A control experiment with toxin previously heated at 100° for 5 min. showed no change. The results (Fig. 1) show that the relative viscosity had fallen nearly to the limiting value before there was an appreciable production of acid-soluble N. After 24 hr. at 25° only 50% of the tropomyosin N was acid-soluble; after dialysis in a cellophan bag at this stage for 72 hr. at 2° against 0.1M-KCl, 70% of the tropomyosin N was still in the bag.

It seemed possible from this experiment that the decomposition was similar to that of myosin by trypsin, in which there is a rapid cleavage of the myosin molecule into two components only, H-meromyosin and L-meromyosin, of very different molecular weight, followed by a slow formation of acid-soluble N, which did not go to completion (Mihályi & Szent-Györgyi, 1953; Mihályi, 1953; Szent-Györgyi, 1953). In two experiments similar to Expt. 1, the digested mixtures and the controls incubated without toxin were examined

in the ultracentrifuge through the kindness of Dr R. A. Kekwick. There is some difficulty in stopping the action of this enzyme instantaneously. The bacterial proteinase is not inhibited by soya-bean trypsin inhibitor; iodo-acetate does not inhibit instantaneously; addition of excess of antitoxin may confuse the analysis; heating the tropomyosin at 100° in the experimental mixture without enzyme itself caused a small decrease in viscosity, e.g. from 2.5 to 2.3. The enzyme action was therefore stopped by cooling to 2° and dialysing, which removed the activator.

Fig. 1. Decomposition of tropomyosin by toxin Oe8a: O, relative viscosity as per cent of original value (2.54); X, acid-soluble N formed as per cent of tropomyosin N.

The digestion was stopped in the first experiment (initial protein, 1%; $\eta_{rel.}$ 5.0) during the rapid phase ($\eta_{rel.}$ 1.8; acid-soluble N, < 10%) and in the second experiment (initial protein, 0.5%; $\eta_{rel.}$ 2.49) during the second phase ($\eta_{rel.}$ 1.09; acid-soluble N, 43%).

I am indebted to Dr Kekwick for his interpretation of these experiments. 'In the ultracentrifuge the tropomyosin showed characteristically a single sharp peak of the type generally displayed by highly asymmetrical molecules, with a sedimentation coefficient of $S_{20w}=2.82$ at a concentration of 0.49 g./100 ml. ($I=0.1$, pH 7.0). After treatment with the enzyme the sharp peak was replaced by a single rather broadly spreading peak having about the same mean sedimentation coefficient. This suggests a general breakdown of the tropomyosin molecule to a rather polydisperse product, which is in complete contrast to the behaviour of myosin towards trypsin (Mihályi & Szent-Györgyi, 1953). In this instance the breakdown of myosin led to the formation of two well-defined components quite different in the ultracentrifuge from myosin itself.'

(2) *Slow phase.* Mihályi (1953) found that the further decomposition of meromyosin by trypsin

was inhibited by the products of the reaction, and that the rate was proportional to the square root of the enzyme concentration. The slow phase of decomposition of degraded tropomyosin by the *Cl. oedematiens* enzyme was examined as follows:

Expt. 2. Toxin Oe8a (150 units, 1.8 mg. N) dissolved in 14 ml. phosphate buffer (pH 7.0, $I=0.1$) and 6 ml. 0.03M cysteine was incubated at 25° with 40 ml. 0.9% tropomyosin (59 mg. N) in 0.1M-KCl. When the relative viscosity had fallen from 3.5 to 1.4, the mixture was rapidly heated in a boiling-water bath and kept there for 6 min. After cooling, the relative viscosity had fallen to 1.22, presumably partly because the enzyme was not inactivated instantaneously. (It was ascertained in a separate experiment that the rate of formation of acid-soluble N was the same in heated and unheated solutions.) The mixture (56 ml.) was dialysed for 20 hr. at 2° against 0.1M-KCl; the recovered solution contained all the original tropomyosin N, of which 10.3% was now acid-soluble. Three flasks A, B and C containing 15.0 ml. of this substrate and respectively 75, 37.5 and 15 units enzyme in 5.0 ml. phosphate-cysteine (pH 7.0) were incubated at 38° and the acid-soluble N was determined at intervals. Fig. 2 shows that at the highest concentration of enzyme more than 90% of the total N had

Fig. 2. Production of acid-soluble N from degraded tropomyosin. Enzyme concn. (units/ml.): A, 3.75; B, 1.87; C, 0.75. Total N 770 µg./ml.

become acid-soluble in 5 hr.; the curves with lower concentrations of enzyme indicate that in time the enzyme becomes inactivated in these conditions. The rates for the first 30 min. were in the ratio 4.2:2.4:1 for enzyme concentrations in the ratio 5:2.5:1; this indicates that the rate is proportional to the enzyme concentration directly, rather than its square root, as the two higher rates were probably not linear for the full 30 min. period. After 22 hr. at 38°, 10 ml. mixture A was dialysed in a cellophan bag at 2° against changes of 0.1M-KCl for 24 hr., at which point 63% of the original tropomyosin N was still in the bag.

In the next experiment repeated additions of toxin were made to find out if the protein could be completely converted into diffusible products:

Expt. 3. Tropomyosin (10.0 ml., 15.2 mg. N) was incubated at 25° with 75 units enzyme in 5.0 ml. SH-buffer (pH 7.0); after 5 hr. another 5.0 ml. of enzyme was added. After 20 hr. the mixture was dialysed for 24 hr. and analysed; it contained 78% of the original protein N. A sample of this solution (7.5 mg. N) was re-incubated at 25° with another 150 units enzyme in SH-buffer for 21 hr., then dialysed and analysed. At least 40% and not more than 55% of the original tropomyosin N was still non-diffusible. Owing to the slow rate and the large correction for the toxin N added a further attempt to determine the end point with crude enzyme seemed unprofitable.

It appears that the decomposition of tropomyosin by *Cl. oedematiens* type B toxin takes place in three stages: (1) a rapid phase, with decrease of viscosity, probably due to a simultaneous attack at several points of the molecule with formation of particles still large enough to be insoluble in trichloroacetic acid; (2) a second phase in which these particles are broken down to particles soluble in trichloroacetic acid with some products diffusible through cellophan; (3) a very slow production of diffusible N from the remainder. The first stage of viscosity reduction is practically completed before the second phase begins; the overlapping in the second and third stages has not been accurately determined. It is not certain that only one enzyme is concerned.

Action of the toxin on (acto-)myosin

The action of the toxin on (acto-)myosin is similar to that on tropomyosin, with a decrease of viscosity in the first phase and then a slow pro-

Fig. 3. Decomposition of myosin and tropomyosin. Toxin Oe8a, 3.45 units/ml., pH 7.0; A, myosin (0.86 mg. N/ml., $I=0.3$); B, tropomyosin (0.91 mg. N/ml., $I=0.1$). Time scale interrupted at 3 hr. ○, viscosity relative to solvent; ●, acid-soluble N as per cent of total.

duction of acid-soluble N. The early phase is shown in Fig. 3. After 20 hr. incubation at 25° the relative viscosity of the (acto-)myosin had fallen to 1.46, compared with 1.1 for tropomyosin, and the acid-soluble N was 25% with (acto-)myosin, against 74% with tropomyosin. On re-incubating these mixtures with additional enzyme (final concentration, 1.5 units/ml.) the viscosity of the (acto-)myosin was not further decreased, but the acid-soluble N rose slowly in 22 hr. to about 50%, compared with over 90% for tropomyosin. At this point about 60% of the (acto-)myosin N was still non-diffusible, of which only about 10% was acid-soluble, while about 40% of the tropomyosin N was non-diffusible but mainly acid-soluble. This experiment compares the extent of the digestion of the two proteins by the enzyme; it is not intended as a comparison of the rate of attack, as at $I=0.1$ tropomyosin still shows some degree of linear aggregation (Tsao, Bailey & Adair, 1951) and the decrease in viscosity may be due partly to some disaggregation consequent on the splitting of peptide bonds.

Properties of the tropomyosinase

The general properties were examined by measurement of the viscosity-reducing action on tropomyosin.

Unit of enzyme activity. Since the purity of the substrate cannot readily be assessed and its viscosity may vary in different batches no attempt was made to define a unit of enzymic activity in terms of the absolute amount of substrate decomposed. For the present experiments it was convenient to designate the stable preparation of 0.02 ml. freeze-dried Oe 8a as a standard containing 3 units and compare other activities with this, using the same preparation of substrate in similar conditions, which varied slightly with the purpose of the experiment. The activity of 1 unit in terms of substrate decomposed may be gauged from the following: 1 unit of Oe 8a in a total vol. 2.5 ml. with 0.01M cysteine at pH 7.0 and 25° and 0.4% tropomyosin decreased the relative viscosity to about 82% of its initial value in 30 min., i.e. approximately 1 unit degraded 2 mg. tropomyosin in these conditions.

Optimum pH. As the viscosity of the tropomyosin mixtures increased slightly with increase in pH, the optimum pH was determined approximately for the crude enzyme by comparison of the time taken by 1 unit of enzyme at a given pH to decompose 25% of the substrate at that pH. Table 1 shows that the optimum was about 7.0 and subsequent experiments were made at this pH.

Effect of enzyme concentration on rate of action. Mixtures were made containing 0.2, 0.4 and 0.8 ml. toxin (6.7 units/ml.) in 0.1M-KCl, 0.25 ml. 0.1M cysteine, 0.45 ml. phosphate buffer (pH 7.0),

0.1M-KCl to total volume 1.5 ml.; and then 1.0 ml. of 1% tropomyosin at 25°. The subsequent decrease in viscosity at 25° is shown in Fig. 4. From these curves Fig. 5 was constructed, showing that

Table 1. *Optimum pH of tropomyosinase*

The mixtures contained 1.0 ml. tropomyosin (1% or 0.75%). 1.0 ml. phosphate buffer ($I=0.1$), 0.25 ml. 0.1M cysteine and 0.25 ml. toxin (1 unit) or 0.1M-KCl.

pH	$\eta_{rel.}$ without toxin at substrate concn.		Time taken with toxin to reduce $\eta_{rel.}$ from (a) to (b) 0.3% concn. (min.)
	0.4% (a)	0.3% (b)	
6.5	2.36	1.89	60
7.0	2.44	1.90	47
7.5	2.54	1.95	55
8.0	2.58	2.03	60

Fig. 4. Time curve of viscosity reduction with different enzyme concentrations. Total vol., 2.5 ml.; tropomyosin, 0.4%; vol. of toxin (6.7 unit/ml.) added: A, nil; B, 0.2 ml.; C, 0.4 ml.; D, 0.8 ml.

Fig. 5. Inverse relation between time required to reach a given viscosity and the enzyme concentration. Data from expt. in Fig. 4. The relative viscosity is marked on each curve.

the time taken to reach a given value of $\eta_{rel.}$ is proportional to the reciprocal of the concentration of the enzyme. With the higher concentrations of enzyme the early readings are more subject to error owing to the rapidity of the change, and with low concentrations the times for the lower $\eta_{rel.}$ values are more subject to error because of the slowness of the rate.

Activation and inhibition. The enzyme was activated by cysteine, glutathione (Fig. 6) and

Fig. 6. Activation of enzyme by sulphhydryl compounds. Enzyme, 1 unit; tropomyosin, 0.37%. A, no activation; B, glutathione 0.004M; C, cysteine 0.01M; D, thiosulphate, 0.01M.

Fig. 7. Reversal of activation with cysteine by iodoacetate. Toxin, 1.0 unit; 0.25 ml. 0.1M cysteine; 1.0 ml. tropomyosin; phosphate buffer to 2.0 ml. initial vol. Incubated at 25°. At arrow (10 min.) 0.5 ml. buffer added to A, and 0.5 ml. 0.1M iodoacetate to B, and the mixtures transferred to viscometer at 25°.

thiolacetate. Thiosulphate did not activate the enzyme but the viscosity of the tropomyosin was decreased in its presence. The activation by cysteine was diminished by iodoacetate, but with a calculated excess the inhibition was not complete (Fig. 7).

Crystalline soya-bean trypsin inhibitor (Worthington Biochemical Sales Co., New Jersey) did not

inhibit the tropomyosinase in a ratio calculated to be at least 6:1 on the polypeptide content ($N \times 6$) of the crude toxin, and presumably much greater on the actual enzyme. This sample of inhibitor had been found to inhibit four times its weight of crystalline trypsin (personal communication from Mrs Sneath).

Action on collagen and red blood cells. Using 'azocoll' as substrate, with a technique similar to that of Bidwell & van Heyningen (1948), no collagenase was detected in the preparations Oe4 and Oe8a; positive results were obtained with smaller amounts of comparable crude *Cl. welchii* toxin. The preparations Oe4 and Oe8a had no haemolytic activity towards horse or sheep cells in phosphate buffer in the presence of cysteine (i.e. in conditions excluding the action of the Ca^{2+} -activated lecithinase); this indicated the absence from these preparations of any SH-activated haemolysin similar to *Clostridium welchii* θ -toxin and of the *Cl. oedematiens* ζ -haemolysin described by Oakley *et al.* (1947).

Antigenic nature of *Clostridium oedematiens* tropomyosinase

Inhibition by antisera. The tropomyosinase activity was inhibited after allowing the enzyme to react with certain horse antisera to *Cl. oedematiens* type B toxins. An antitoxic serum (no. 1141 from the Wellcome Research Laboratories) was designated as a standard serum containing 6000 units of antitropomyosinase/ml. and the relative titres of other sera were determined in the following way.

Toxin and antisera were dissolved or diluted in phosphate buffer (pH 7.0, $I=0.1$). In a series of tubes the test dose of toxin Oe8a (1 unit tropomyosinase in 0.5 ml.) + 0.25 ml. 0.1M cysteine was mixed with antitoxin and the volume made up to 1.5 ml. with buffer. After 25 min. at room temperature the tubes were placed at 25° for 5 min.; 1.0 ml. 1% tropomyosin in 0.1M-KCl was added, and the mean residual enzyme activity determined, in relation to that of the test dose without any antitoxin, by the times required to decrease the viscosity to three or four successive levels, e.g. from initial $\eta_{rel.}$ 2.4 to 2.2, 2.1, 2.0 and 1.9. The curve relating residual activity to antitoxin units was approximately linear and reasonably reproducible between 0.7 and 1.4 units antitoxin. In seven separate tests, the residual activity of 1 unit of enzyme (Oe8a) after combination with 1 unit antitropomyosinase (Serum 1141) was 0.54, 0.58, 0.58, 0.34, 0.58, 0.58 and 0.62 unit. With lower or higher amounts of antitoxin (less than 0.6 or more than 1.4 units) the results were less reproducible, presumably owing to the errors in the steep or flat part of the activity curve respectively. In practice the titre of an unknown serum was first determined approximately and redetermined within the range 0.7–1.4 units by reference to the standard antitoxin curve.

Inspection of the titres of various sera (Table 2) shows first that there is no correlation between the

antitropomyosinase and the anti- α -toxin titres; and secondly that there is no correlation between the antitropomyosinase and the anti- ζ -toxin titres. All the antisera to *Cl. oedematiens* type B toxins contained some antitropomyosinase, although the titres of the two sera produced against the type B Todd strain were relatively low; a sample of *Clostridium haemolyticum* antitoxin was also inhibitory. The *Cl. oedematiens* International Standard antitoxin and the type A antitoxin 644 and *Cl. welchii* antitoxin had no demonstrable antitropomyosinase; because of the increase in initial

viscosity of the test mixtures by the addition of glycerol (International Standard) or serum protein the limiting values of low-titre sera could not be determined accurately.

Tropomyosinase content of various crude toxins

The tropomyosinase content of different preparations was estimated by comparing the activity of a sample with that of 1 unit of the standard Oe 8a in conditions similar to those for the antitoxin titration, but omitting the 25 min. period for combination. The results in Table 3 show that the

Table 2. *Titres of specific antitoxins in antitoxic horse sera*

The antitropomyosinase (anti- η) titres were determined against a test dose of *Cl. oedematiens*, type B toxin Oe 8a.

Immunizing toxin	Source	No.	Sera		
			Titres of specific antitoxin (units/ml.)		
			Anti- η *	Anti- α †	Anti- ζ ‡
<i>Cl. oedematiens</i> Type B, Albiston	W.R.L.	1141	6000	1000	5000
	W.R.L.	2209	280	1200	2500
	W.R.L.	1152	4800	1000	3000
	L.I.P.M.	77	8000	3000	—
	L.I.P.M.	Monospecific§	540	1000	—
Type A + B	W.R.L.	850	1650	1750	5000
Type B, Todd	W.R.L.	T3	1600	6250	110
	W.R.L.	T61	ca. 80	1650	150
Type A	R.W.L.	644	(Nil)	800	140
	M.R.C.	Standard	(Nil)	20	—
<i>Cl. haemolyticum</i>	W.R.L.	EX 1250	140	—	—
<i>Cl. welchii</i>	L.I.P.M.	RP 586§	(Nil)	2500	—
	L.I.P.M.	Monospecific§	(Nil)	1000	—

W.R.L. = Wellcome Research Laboratory. L.I.P.M. = Lister Institute of Preventive Medicine.
M.R.C. = National Institute for Medical Research, Mill Hill.

* Serum no. 1141 as standard. † Anti-lethal *Cl. oedematiens* i.u. ‡ Wellcome units.
§ Pepsin-refined antitoxin. || *Cl. welchii* antitoxin i.u.

Table 3. *Tropomyosinase content of various crude toxins*

Kind of toxin			No.	Units tropomyosinase	Source of toxin
Organism	Type	Strain			
<i>Cl. oedematiens</i>	B	Albiston	Oe 7	0.92/mg.	L.I.P.M.
			Oe 8	1.20/mg.	
			OC12345	0.88/mg.	
			OK 15145	0.42/mg.	
			XE 1074	0.33/mg.	
	B	<i>gigas</i>	12XB	Nil in 7.0 mg.	W.R.L.
	B	Todd	AE 1258*	Nil in 1.0 ml.	
				Nil in 10 ml. after concentration	
				0.02/mg.	
	A	—	OC 19345	Nil in 20 mg.	
<i>Cl. haemolyticum</i>	A	—	Test toxin	Nil in 10 mg.	M.R.C.
	A	—	Evans	Nil in 10 mg.	D. G. Evans
	—	—	NX 605	0.05/mg.	W.R.L.
			HC 231048	0.02/mg.	

* Liquid culture filtrate.

crude toxins of *Cl. oedematiens* type B, strains Albiston or *gigas*, are the highest in activity. No activity was found in the type A toxin, NX 510, nor in two other *Cl. oedematiens* toxins, the M.R.C. test toxin and one given by Dr D. G. Evans, which are probably also type A, nor was any activity found in various samples of *Cl. welchii*, *Clostridium septicum* and *Corynebacterium diphtheriae* toxins.

The *Cl. haemolyticum* toxin tested had some activity. The relative inhibitory activity of the *Cl. oedematiens* antiserum no. 2209 and the *Cl. haemolyticum* antiserum EX 1250 against a test dose of the standard toxin Oe 8a and a test dose of the *Cl. haemolyticum* toxin, each containing 0.5 unit enzyme, were about equal. It seems probable by the equality of the cross-neutralization that the antigens are identical. This is of some taxonomic interest, as *Cl. oedematiens* type B and *Cl. haemolyticum* toxins also possess the β -antigen (lecithinase) in common (Oakley *et al.* 1947; Macfarlane, 1950).

Designation of the tropomyosinase

It is concluded that the tropomyosinase in the *Cl. oedematiens* type B filtrates is a specific antigen distinct from any of those designated by Oakley *et al.* (1947), on the following grounds: (1) By the lack of correlation in antitoxin titres of different antisera, it is distinct from the α - and ζ -toxins. (2) It is also distinct from the α -toxin, because it was not detected in the M.R.C. toxin used for titration of the International Standard antitoxin, nor in the lethal type A toxin NX 510. (3) It is distinct in biochemical activity from the β - and γ -antigens, which are lecithinases, and from the α - and ζ -antigens, which are haemolytic. (4) It is distinct by distribution and activity from the ϵ -antigen, which, possibly as a lipase, gives rise to a pearly layer in egg yolk and which is found in type A but not in type B culture filtrates.

The antigen has therefore been designated as *Cl. oedematiens* η -antigen. From the biochemical point of view, it seems convenient to name the enzyme in the meantime as a tropomyosinase until its substrate specificity is clearly defined.

DISCUSSION

The formation *in vivo* of an enzyme which decomposes (acto-)myosin and tropomyosin might be of some significance in disease caused by *Cl. oedematiens*. Curiously enough, tropomyosinase does not appear to be produced by type A strains, which in man cause gas gangrene due to infection of muscle, but it is produced by type B strains which cause infectious necrotic hepatitis (black disease, German braxy) in sheep; in this disease the organ-

isms multiply in the liver, in necrotic areas produced by invasion with liver flukes (Turner, 1930). There is little doubt that the main lethal component in type A and type B toxins produced *in vitro* is the classical α -toxin, but the significance in infections of the other antigens, including the tropomyosinase, has not been assessed.

It does not appear that the presence of any proteinase in *Cl. oedematiens* filtrates has previously been established. Evans (1947) examined filtrates from a number of strains for collagenase and gelatinase but, as in the present work, with negative results. Taxonomically it is of interest that the *Cl. haemolyticum* toxin and antitoxin examined contained tropomyosinase and the anti-enzyme respectively, and that cross-neutralization tests indicate that the *Cl. haemolyticum* and *Cl. oedematiens* antigen are identical. Oakley *et al.* (1947) pointed out that the possession in common of the β -antigen supported the view that *Cl. haemolyticum* is a member of the *oedematiens* group, and this view is again strengthened by the common possession of the η -antigen.

The crude tropomyosinase preparations decompose tropomyosin in at least two distinct stages, characterized by a rapid decrease in viscosity and a slower but complete decomposition to particles soluble in trichloroacetic acid. It also decomposes (acto-)myosin with production of at least 50% of acid-soluble nitrogen. Bailey (1948) suggested that tropomyosin may be one of the ultimate units of which myosin is composed, though it is certain that myosin consists of something more than tropomyosin; for one thing tryptophan is present in myosin but not in tropomyosin. The present findings that (acto-)myosin and tropomyosin are both decomposed by the bacterial preparation is at least not inconsistent with Bailey's suggestion, and the experiments indicated the possibility of a completely or relatively resistant 'core' in (acto-)myosin. The end point of this action was not established, however, nor has it yet been shown that the decomposition of tropomyosin and (acto-)myosin is due to the same or to a single enzyme. The tropomyosinase may be a useful tool in the elucidation of the structure of these proteins, but considerable purification of the enzyme would first be necessary.

SUMMARY

1. Certain crude toxins of *Clostridium oedematiens* decompose tropomyosin and myosin.

2. The decomposition of tropomyosin takes place in three stages characterized by: (1) rapid decrease in viscosity; (2) production of particles soluble in trichloroacetic acid but not diffusible through cellophane; (3) slow production of diffusible particles.

3. The viscosity-reducing action is due to a sulphhydryl-activated enzyme, which has been named a tropomyosinase, whose action is inhibited by homologous antisera.

4. The enzyme does not attack collagen, nor haemolyse the red blood cells of sheep and horses. It is activated by cysteine, glutathione and thiolacetate, but not by thiosulphate. It is not inhibited by soya-bean trypsin inhibitor.

5. By neutralization tests with selective antisera, this tropomyosinase was identified as a specific antigen which has been designated, in accordance with the nomenclature of Oakley *et al.* (1947), as *Cl. oedematis* η -antigen. It has been found in crude toxins of *Cl. oedematis* type B and *Clostridium haemolyticum*, but not in *Cl. oedematis* type A nor in *Clostridium welchii*, *Clostridium septicum* or *Corynebacterium diphtheriae* toxins.

I wish to express my thanks to my colleagues Mr Weitz and Dr Dolby, to Miss M. Barr, Dr D. A. Long, Dr D. G. Evans and especially to Dr G. H. Warrack of the Wellcome Research Laboratories for gifts of toxins and antitoxins, to Miss S. Jolley for her assistance, and to Mr H. Murray for the ultracentrifugal examinations.

REFERENCES

- Bailey, K. (1942). *Biochem. J.* **36**, 121.
Bailey, K. (1948). *Biochem. J.* **43**, 271.
Bailey, K. (1951). *Biochem. J.* **49**, 23.
Bailey, K. (1954). In *The Proteins*, 2, B, 959 (eds. Neurath, H. & Bailey, K.). New York: Academic Press Inc.
Bidwell, E. & Van Heyningen, W. E. (1948). *Biochem. J.* **42**, 140.
Evans, D. G. (1947). *J. gen. Microbiol.* **1**, 378.
Lewis, G. M. & Macfarlane, M. G. (1953). *Biochem. J.* **54**, 138.
Macfarlane, M. G. (1950). *Biochem. J.* **47**, 267.
Mihályi, E. (1953). *J. biol. Chem.* **201**, 197.
Mihályi, E. & Szent-Györgyi, A. G. (1953). *J. biol. Chem.* **201**, 189.
Miles, A. A. & Miles, E. M. (1952). *J. Physiol.* **118**, 228.
Oakley, C. L., Warrack, G. H. & Clarke, P. H. (1947). *J. gen. Microbiol.* **1**, 91.
Szent-Györgyi, A. G. (1953). *Arch. Biochem. Biophys.* **42**, 305.
Tsao, T.-C., Bailey, K. & Adair, G. S. (1951). *Biochem. J.* **49**, 27.
Turner, A. W. (1930). *Aust. Counc. Sci. Indust. Res. Bull.*, no. 46.

The Interconversion of Serine and Glycine: Participation of Pyridoxal Phosphate

By R. L. BLAKLEY

*Department of Biochemistry, John Curtin School of Medical Research, Australian National University,
Canberra, Australia*

(Received 22 March 1955)

The enzyme system of avian and mammalian liver which catalyses the reversible synthesis of serine from glycine and formaldehyde has been shown to require tetrahydropteroylglutamic acid (tetrahydroPGA) as a coenzyme (Blakley, 1954*b, d*; Kisliuk & Sakami, 1954). In well dialysed, unfractionated liver extracts pteroylglutamic acid (PGA) and some other derivatives of it besides tetrahydroPGA also stimulate serine synthesis (Blakley, 1954*a, d*), but the evidence indicates that in such crude enzyme preparations PGA and derivatives of it other than tetrahydroPGA are first converted enzymically into the latter compound before participating in serine biosynthesis (Blakley, 1954*d*). When crude enzyme preparations are used diphosphopyridine nucleotide (DPN) and adenosine triphosphate (ATP) in conjunction with PGA also stimulate serine-glycine interconversion, but the effect of ATP and DPN

appears to be concerned with the transformation of PGA into tetrahydroPGA (Blakley, 1954*d*). Thus the only coenzyme of serine biosynthesis, for which *in vitro* studies by the isotope method have provided conclusive evidence, is tetrahydroPGA. *In vivo* experiments have suggested, however, that pyridoxal phosphate also participates in serine synthesis (Deodhar & Sakami, 1953).

Serine biosynthesis in the presence of a partially purified enzyme system has now been studied by a manometric method, and under appropriate conditions a marked activation by pyridoxal phosphate has been observed (Blakley, 1954*c*). In the present communication the evidence for the activation of enzymic serine synthesis by pyridoxal phosphate is presented in detail, together with data concerning the effect on the reaction of other pyridoxine derivatives.