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Purification and Partial Characterization of the Exotoxin of *Corynebacterium ovis*

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1. The toxin from *Corynebacterium ovis*, a phospholipase D (sphingomyelin phosphodiesterase D) that acts on 2-lysophosphatidylcholine and sphingomyelins, was purified by about 400-fold to homogeneity as judged by several criteria. [The EC number of the toxin (EC 3.1.4.41) has been allotted by the Nomenclature Committee of IUB, but has not yet been published.] 2. A new assay method performed *in vitro*, based on inhibition by the toxin of erythrocyte lysis by staphylococcal β-haemolysin, was developed to facilitate the purification. 3. The toxin was found to be a basic (pI 9.1) glycoprotein of mol.wt. 14500 ± 1000. 4. The amino acid composition of the toxin was highly reminiscent of that of collagen, since it contained hydroxyproline, hydroxylysine and a high proportion of glycine, but preliminary tests showed no other similarities to collagen or proteins with similar compositions.

Corynebacterium ovis is a bacterial pathogen causing suppurative infections mainly in sheep, goats and horses and occasional infections in other species (Carne, 1939). It produces a powerful exotoxin *in vitro*, but unlike the situation with *Corynebacterium diphtheriae* there is no clinical evidence of acute or chronic poisoning in natural infections. The lesions caused by the bacteria are characteristically pyogenic, with suppuration and abscess formation at the initial sites of infection often followed by secondary abscess formation in the regional lymph nodes and sometimes internal organs. Toxinogenesis and pyogenesis are two distinct properties; toxin production is believed to be phage-dependent (H. R. Carne, unpublished work).

The general properties of the crude exotoxin have been reported by Carne (1940) and Jolly (1965). The major role of *C. ovis* toxin in natural infection seems to be the facilitation of the spread of the causative bacteria by its action as a permeability factor. It thus causes marked leakage of plasma from small blood vessels at the site of infection, which floods lymphatic spaces and increases the risk of bacteria being carried to the regional lymph nodes.

Souček *et al.* (1967) reported that the toxin of *C. ovis* was a phospholipase D acting on sphingomyelin to produce *N*-acylsphingosyl phosphate (ceramide phosphate) and choline. A partial purification has been described (Souček *et al.*, 1971; Souček & Součkova, 1974; Goel & Singh, 1972).

Zaki (1965) demonstrated that the lysis of ox erythrocytes by staphylococcal β-haemolysin could be inhibited by the products of toxinogenic strains of *C. ovis*. It was found that this inhibiting effect paralleled the toxicity as measured *in vivo*. The effect *in vitro*, thought to be caused by the toxin, is believed to be the result of its action on the sphingomyelin molecules, rendering them unsusceptible to subsequent attack by the phospholipase C activity of the staphylococcal β-haemolysin. This action has been investigated further by Souček & Součkova (1974) and Carne & Onon (1978).

Observations on the nature of the anatomical lesions produced by the toxin had suggested that the primary sites of action of the toxin were the walls of the blood vessels. Particularly suggestive were the experimental observations by Jolly (1965), who labelled plasma with Evans Blue and observed areas of blueing at sites of intradermal inoculation of toxin, which were proportional to the doses of toxin injected. Furthermore, injection of toxin into the vascular corneosclerotic junction in sheep produced a severe local reaction similar to that seen in the skin, whereas injection into the centre of the avascular cornea produced no significant effect.

These findings, coupled with the observations *in vitro* of the toxin on erythrocytes mentioned above, led to the speculation that the primary biochemical target of the toxin is the sphingomyelin in the cell membrane of endothelial cells lining blood vessels, and evidence to support this has been presented elsewhere (Carne & Onon, 1978).

The present study was undertaken to produce a pure compound in order to determine the mode of action of the toxin and to investigate the nature of

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the biochemical lesion it produces. The purification procedures and chemical properties of the toxin together with a new method of assaying its activity *in vitro* are described here.

Materials and Methods

All reagents were of analytical grade. DEAE-cellulose (DE-52) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K.; CM-50 Sephadex was obtained from Pharmacia (G.B.) Ltd., London W5 5SS, U.K.

Production of toxin

C. ovis was grown in a special medium of the following composition: brain/heart infusion, 37g (Difco Laboratories, West Molesey, Surrey, U.K.); yeast extract, 5g (Difco); lactalbumin hydrolysate, 10g [Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.]; NaCl, 5g; water, 1000ml. The solution was adjusted to pH 7.6, distributed in 300ml volumes in circular penicillin flasks and autoclaved at 82.7kPa (12lbf/in^2) for 12min. The flasks were sown with exponential-phase cultures grown on tryptic digest/agar and incubated at 37°C for 20h in a horizontal shaker. The culture was centrifuged (118000*g*) and the supernatant passed through a 22μm Millipore filter. The bacteria-free filtrate is hereafter referred to as 'toxic filtrate' (TF).

Assay of toxin

Toxic filtrate and all major toxic fractions were assayed *in vivo* by intradermal injection in rabbits with 0.2ml of logarithmic dilutions. The MRD (minimal reacting dose) was that which produced an area of congestion and slight thickening of the skin 1.0cm in diameter. Doses above this produced increasing areas of swelling and degrees of congestion. In the higher range of doses a central area of necrosis developed and the surrounding oedema became progressively more marked.

The fractions from the various columns used in the purification were assayed *in vitro* to locate toxic activity. The method *in vitro* is based on the fact that staphylococcal β-haemolysin will cause the lysis of sheep erythrocytes if incubated successively with them at 37 and 4°C. This effect can be inhibited if the cells are first exposed to *C. ovis* toxin. Agar (1% Oxoid no. 1; Oxoid Ltd., Basingstoke, Hampshire, U.K.; or 1.5% Lab M; Lab M, London EC3R 7QJ, U.K.) was made up in phosphate-buffered saline (Oxoid Dulbecco A tablets with 4mM-$MgCl_2$, pH 7.2; Dulbecco & Vogt, 1954) containing 0.01% NaN_3 and poured (10ml) into standard (8cm-diameter) disposable Petri dishes (Sterilin, Teddington, Middx., U.K.). When cold, a second layer containing the same ingredients with the addition of 5% sheep blood was poured on top. The plates were kept at 4°C until required and could be stored for about 3 weeks. Wells were cut into the agar with a cork borer or commercial well cutter (0.5cm diameter), and 50μl samples of the various fractions incubated in the wells overnight at 37°C. On the following day each plate was covered with 1ml of staphylococcal β-haemolysin prepared by the method described by Lovell & Zaki (1966) and incubated for 1h at 37°C, followed by 1h at 4°C. The diameter of the circle of inhibition of lysis is proportional to the concentration of toxin (see Fig. 1). The activity *in vitro* of the toxin is expressed in terms of MRD, 1 MRD being the amount of material per ml causing a circle of inhibition with a diameter of 1.5cm (the well diameter of 0.5cm being included in this value). By using a single large plate of uniform agar thickness, all the fractions off a single purification column can be assayed, thus increasing the accuracy of the assay. However, it was found more convenient to use a number of 8cm disposable plates with six to eight wells for routine location of activity, provided that the plates were all of the same batch.

Purification steps

$(NH_4)_2SO_4$ precipitation. The toxic filtrate was adjusted to 35% satn. with $(NH_4)_2SO_4$. The precipitate collected by centrifugation was discarded and $(NH_4)_2SO_4$ was dissolved in the supernatant to 65% satn. The resulting precipitate was either force-dialysed against water and freeze-dried or dialysed

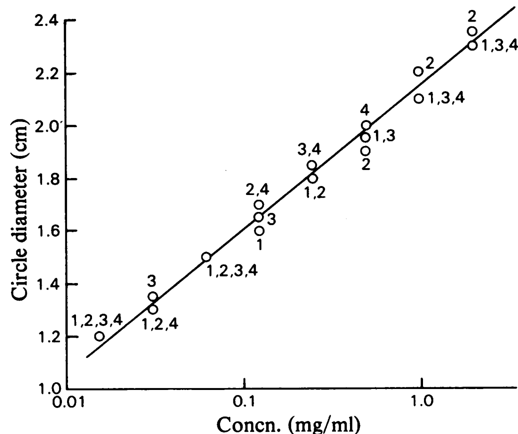

Fig. 1. *Dose–response curve of toxin measured in vitro* Various doses of the most highly purified fraction (1DE) were assayed by measuring the diameter of the circle over which they inhibited lysis of sheep erythrocytes by staphylococcal β-haemolysin after diffusion from wells cut in the agar plate. The assay was carried out four times in plates marked 1–4, and these numbers are shown against the diameters obtained for each concentration of toxin.

overnight against the column buffer used in the next step of purification. The active fraction from the $(NH_4)_2SO_4$ precipitation is designated '35–65'.

Ion-exchange chromatography. The 35–65 fraction was applied to a column (2cm × 9cm) of Sephadex CM-50 ion-exchanger buffered with 0.1M-sodium acetate, pH 6.1, by the method described by Souček & Součkova (1974). A flow rate of 30ml/h was maintained. After the unbound material had been eluted, a linear salt gradient (500ml of buffer and 500ml of buffer containing 2M-NaCl) was applied. Despite the low buffering capacity of the acetate, no sudden change of pH occurred when the toxin emerged. The active fraction from this step, designated 'CM3', was force-dialysed and freeze-dried. The elution profile is shown in Fig. 2: a small peak (CM1) of activity emerged with the unbound material.

Fraction CM3 was applied to a column (2cm × 9cm) of DEAE-cellulose exchanger buffered with 0.05M-Tris/HCl, pH 8.0, and maintained with a flow rate of 30ml/h. As before, a linear salt gradient was applied after all the unbound material had emerged from the column. The active fraction (1DE) was force-dialysed and freeze-dried.

Isoelectric focusing. Fraction 1DE was applied to an analytical isoelectric-focusing slab gel ('PAG plate') as described in the LKB instruction manual (see below), with a slight modification. The running time was lengthened (from 1½ to 2½h) and the voltage decreased (from 1200 to 600V). After focusing, the gel was sectioned, eluted with phosphate-buffered saline and assayed. The active fraction was designated E1. Section gels were also eluted with water and the pH of the eluate was measured.

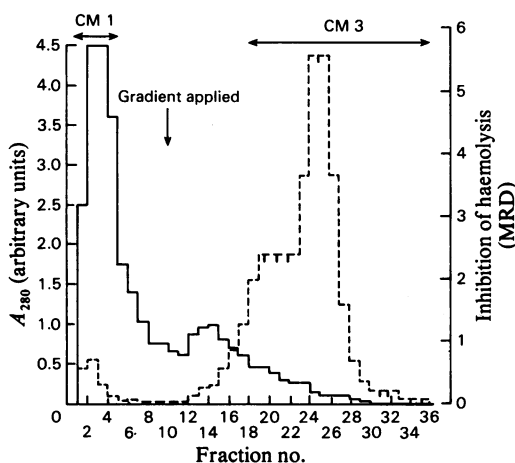

Fig. 2. *Elution profile of fraction* 35–65 *on CM-Sephadex*
The active fraction (35–65% saturation) from $(NH_4)_2SO_4$ precipitation was applied to a column of CM-Sephadex CM-50. ——, A_{280}; ----, inhibition of haemolysis (MRD protein). The column (9cm × 2cm) was equilibrated with 0.1M-sodium acetate, pH 6.1, and eluted with a linear salt gradient over 1 litre to a solution of 0.1M-sodium acetate, pH 6.1, and 2M-NaCl.

Criteria of purity

N-Terminal analysis. Fractions 1DE and E1 were analysed by Hartley's (1970) method.

Disc electrophoresis. Fraction 1DE was subjected to electrophoresis with the β-alanine system described by Pearce *et al.* (1972). Urea was used instead of sucrose in order to make the samples dense.

Isoelectric focusing. Fraction 1DE was focused in slab gels as above and in rod gels as described by Pearce *et al.* (1972).

Immunodiffusion precipitation. Fraction 1DE was allowed to diffuse against antiserum raised against crude toxic filtrate by the method described in the LKB handbook supplied with their equipment for microscope-slide double-diffusion precipitin reactions (LKB Produkter AB, Stockholm, Sweden; Operation Manual 6800A).

Protein determination

The method described by Lowry *et al.* (1951) was used. In addition measurement of A at 210nm (Tombs *et al.*, 1959) and the biuret method were also used. Electrophoretically pure (A grade) bovine serum albumin (Calbiochem, San Diego, CA, U.S.A.) was used in each method to draw up the calibration curve.

Carbohydrate analysis

The carbohydrate content of fraction 1DE was estimated by the method described by Clamp *et al.* (1971) on a Pye–Unicam g.l.c. machine. The methylsilyl derivatives were prepared and run as described, with mannitol as the internal standard.

Results

Purification and degree of purity

Table 1 shows a typical purification of toxic filtrate. Preliminary tests showed that most of the activity measured *in vivo* and *in vitro* was located in the 35–65%-satd.-$(NH_4)_2SO_4$ fraction, the second half of the second peak to emerge from the CM-Sephadex column and the first (unbound) peak from the DEAE-cellulose column. Isoelectric focusing was not found to be a satisfactory purification step since a great deal of activity was lost. The activity was found in the basic region of the isoelectric-focusing gel (pH 9.1) and protein eluted from this region was active *in vitro* and *in vivo*. Staining the commercial 'PAG plate' with Coomassie Blue showed a single band at the basic end of the gel. Staining for carbohydrate with periodate/Schiff reagent showed a faint band corresponding to the protein band.

Table 1. *Purification of C. ovis toxin*

The fractions are those described in the Materials and Methods section, where the purification procedure is also given. 1 MRD is the amount of material (per ml) causing a circle of inhibition of lysis with a diameter of 1.5cm.

Fraction	Weight (mg)	Protein (mg/mg dry wt.)	MRD (mg)	MRD (mg of protein)	Total protein (mg)	Total MRD	Recovery (%)	Purification (-fold)
TF	26500	0.438	15.000	6.570	11607	1767	100	1
35–65	800	0.600	0.500	0.300	480	1600	91	22
CM3	216	0.058	0.290	0.017	12.5	735	42	386
1DE	40	0.241	0.063	0.015	9.6	640	36	438

N-Terminal analysis of fractions 1DE and E1 showed that both samples were reasonably pure. Fraction 1DE showed only one very strongly fluorescing spot corresponding to arginine. Fraction E1 also showed only one strongly fluorescing spot corresponding to arginine, but very faint traces of proline, isoleucine, phenylalanine and glycine were also present. These very faint spots may have been due to contamination with the marker amino acids.

Disc electrophoresis of fraction 1DE showed only one protein-staining band in the middle of the gel when Coomassie Blue was used as the stain. When Amido Black was used there was an additional diffusely staining band at the bottom of the gel beyond the Neutral Red tracker dye. This difference may be related to the fact that Amido Black stains carbohydrates and proteins, whereas Coomassie Blue stains proteins only.

Immunodiffusion precipitation of fraction 1DE against antiserum raised against crude toxin gave only one precipitin line.

Protein estimation

The protein content of each fraction from the purification procedure was measured by three different methods with bovine serum albumin as the standard. The methods used yielded protein concentrations lower than had been expected, considering the purification steps carried out and the degree of purity of fraction 1DE. Lipids, salts and carbohydrate did not appear to be present in significant amounts to account for the low percentage protein in fraction 1DE.

Fraction 1DE was found to be hygroscopic and, although the protein estimations were carried out on material weighed immediately after freeze-drying, there may have been a rapid absorption of water. This factor, too, might partly explain the low percentage of protein obtained.

Amino acid composition

The amino acid analysis was carried out in the customary manner in a Chromaspeck analyser with norleucine as the internal standard. Table 2 shows the results from the analysis of fraction 1DE. The unusual composition of a high content of glycine, proline and hydroxyproline and the presence of hydroxylysine is highly reminiscent of collagen. This led to a few preliminary experiments that have shown that the toxin did not lose activity when treated with a highly purified collagenase [clostridiopeptidase; Boehringer Corp. (London) Ltd., London W5 2TZ, U.K.], did not cross-react with antiserum (Mercia Diagnostic Ltd., Watford, Middx., U.K.) against subcomponent C1q (a gift from Dr. R. Clague, Department of Rheumatology, University of Manchester), a protein containing the typical collagen amino acids, had no collagenous structure when observed with the electron microscope and had no collagenase or gelatinase activity (Weiss, 1976).

The low tyrosine content would account for the low protein estimation obtained with the Lowry method, and the high percentage of proline and

Table 2. *Amino acid composition of C. ovis toxin*

Amino acid	Composition (residues/1000 residues)
Hyp	50
Asp	45
Thr	21
Ser	62
Glu	66
Pro	143
Gly	314
Ala	91
Val	24
Met	7
Ile	19
Leu	22
Tyr	8
Phe	12
Hyl	14*
Lys	33
His	25
Arg	65

* Separate analysis.

Table 3. *Carbohydrate content of fraction 1DE*
The carbohydrate content of fraction 1DE, believed to be pure toxin, was analysed as described in the text with mannitol as the internal standard. Protein was measured by the method of Lowry *et al.* (1951).

Sugar	Content (μmol/mg of sample)
Mannose	0.0328
Galactose	0.1634
Glucose	0.0596
N-Acetylglucosamine	0.0744
N-Acetylgalactosamine	0.0572
Total	0.3874
Percentage of sample (w/w)	7
Percentage of protein (w/w)	29

hydroxyproline would explain the low readings obtained with the biuret reagent.

Carbohydrate content

The carbohydrate composition of the toxin is shown in Table 3. No unusual sugars were found, and the five that were detected represent 7.0% of the dry weight of fraction 1DE or 29% of the estimated protein present. It is not possible to reconcile the difference in weight between the dry material and protein content in terms of carbohydrate. This left the possibility of lipid accounting for this difference. However, extraction of fraction 1DE with lipid solvents (chloroform/methanol, 2:1, v/v) resulted in loss of activity, but no appreciable loss of material, implying that lipids were not responsible for the difference in weight.

Molecular weight

The molecular weight of the toxin was calculated from the sedimentation coefficients (corrected for water at 20°C) by assuming a partial specific volume of 0.735 g/ml and a frictional ratio of 1.15. Values of 1.83 S and 1.76 S were obtained from the ultracentrifuge runs. Allowing for some asymmetry and hydration, the molecular weight was estimated to be 14500 ± 1000.

Discussion

The method of assay of *C. ovis* toxin *in vitro* described above was found to be more convenient than that described by Souček *et al.* (1962). The method described in the present paper enables fractions to be assayed directly off the column without the need for dilution or concentration.

The method *in vitro* was also found to be a true reflection of toxicity. This is shown by the fact that the single protein produced by isoelectric focusing of fraction 1DE was active *in vivo* as well as causing inhibition of the staphylococcal β-haemolysin in the *in vitro* system. These two effects paralleled one another in degree according to the concentration of the sample.

A 400-fold purification was achieved by the methods described above, and by several criteria (electrophoresis, *N*-terminal analysis and immunodiffusion precipitation) fraction 1DE was deemed to be free from other proteins. This compares with the 176-fold purification reported by Souček *et al.* (1971), who used methanolic precipitation and ion-exchange chromatography on CM-50 Sephadex, and the 3-fold purification achieved by Goel & Singh (1972) by using $(NH_4)_2SO_4$ precipitation and gel filtration on Sephadex G-100.

As Souček *et al.* (1971) had found, toxin was eluted from the CM-50 Sephadex column in two peaks. We think that the first peak of activity is caused by a displacement of the toxin by a front of changed conditions. Re-running of this initial peak on a fresh column causes the toxin to bind and it can be eluted in the same position as the second peak of activity when a salt gradient is applied. In my hands, Sephadex G-100 was unsatisfactory, since the activity was spread throughout the column and could not be totally confined to one peak even by the addition of urea or alcohol. This anomalous behaviour of the toxin prevented a molecular-weight estimation on this material, but suggested that the toxin might be a glycoprotein. This was also suggested by the Amido Black staining of fraction 1DE on disc gels and the periodate/Schiff-positive band on isoelectric-focusing gels. Indeed five sugars were found on analysis of fraction 1DE, the total carbohydrate constituting 7.0% of the weight of fraction 1DE or 29% of the protein determined by Lowry's method.

The difference in weight between the dry toxin (1DE) and the estimated protein weight could not be adequately explained. Salts, lipids and carbohydrates were not present in significant amounts. It is possible that the toxin was absorbing moisture from the atmosphere, since it was noticed that fraction 1DE was hygroscopic, but the amino acid composition of the toxin probably explains the low readings.

As mentioned above, the amino acid composition of the toxin is extremely unusual in having the collagenous constituents hydroxyproline, proline, hydroxylysine and a high glycine content. Apart from the collagenous proteins, only the C1q subcomponent of complement (Reid *et al.*, 1972), conglutinin (an immunological factor found only in ruminants; Lachmann, 1967) and acetylcholinesterase (Rosenberry & Richardson, 1977) have similar amino acids. Both subcomponent C1q and acetylcholinesterase have specific regions of the molecule that are collagenous in amino acid composition. Neither collagen nor conglutinin had an inhibitory action on staphylo-

coccal β-haemolysin characteristic of *C. ovis* toxin and used for titration *in vitro*. Unlike collagen etc., the toxin from *C. ovis* has a low molecular weight and, as might be expected from its weight, no fibrous or structured elements when examined with the electron microscope. Bacterial collagenase (proteinase-free) caused no loss of activity or physical breakdown of the toxin, and immunoelectrophoresis against anti-(subcomponent C1q) serum showed no lines of identity. This preliminary evidence suggested that the toxin had little in common with the typical collagen triplet sequence (Gly-Pro-Y or Gly-X-Hyp), but it is difficult to see how the high proportions of the collagenous amino acids of the toxin could be arranged in such a fashion as not to be susceptible to attack by the clostridiopeptidase (bacterial collagenase).

The molecular weight of the toxin, namely 14500, agrees with that determined by Souček & Součkova (1974), who suggested 16000–18000 from use of thin-layer gel filtration. This low molecular weight agrees with the apparent diffusion of the toxin out of polyacrylamide gels. However, aggregation may occur under some conditions (A. Souček, personal communication to H. R. Carne), since in trial runs some toxin was eluted from Sephadex G-100 early.

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References

Carne, H. R. (1939) *J. Pathol. Bacteriol.* **49**, 313–328

Carne, H. R. (1940) *J. Pathol. Bacteriol.* **51**, 199–212

Carne, H. R. & Onon, E. O. (1978) *Nature (London)* **271**, 246–248

Clamp, R. J., Bhatti, T. & Chambers, R. E. (1971) *Methods Biochem. Anal.* **19**, 229–344

Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182

Goel, M. C. & Singh, I. P. (1972) *J. Comp. Pathol.* **82**, 345–353

Hartley, B. S. (1970) *Biochem. J.* **119**, 805–822

Jolly, R. D. (1965) *J. Comp. Pathol.* **75**, 417–431

Lachmann, P. J. (1967) *Adv. Immunol.* **6**, 479–527

Lovell, R. & Zaki, M. M. (1966) *Res. Vet. Sci.* **7**, 307

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Pearce, F. L., Banks, B. E. C., Banthorpe, D. V., Berry, A. R., Davies, H. ff. S. & Vernon, C. A. (1972) *Eur. J. Biochem.* **29**, 417–425

Reid, K. B. M., Lowe, D. M. & Porter, R. R. (1972) *Biochem. J.* **130**, 749–763

Rosenberry, T. L. & Richardson, J. M. (1977) *Biochemistry* **16**, 3550–3557

Souček, A. & Součkova, A. (1974) *J. Hyg. Epidemiol. Microbiol. Immunol.* **18**, 327–335

Souček, A., Součkova, A., Mara, M. & Patočka, F. (1962) *J. Hyg. Epidemiol. Microbiol. Immunol.* **6**, 13–23

Souček, A., Michalec, C. & Součkova, A. (1967) *Biochim. Biophys. Acta* **144**, 180–182

Souček, A., Michalec, C. & Součkova, A. (1971) *Biochim. Biophys. Acta* **227**, 116–128

Tombs, M. P., Souter, F. & MacLagan, N. F. (1959) *Biochem. J.* **73**, 167–171

Weiss, J. (1976) *Int. Rev. Connect. Tissue Res.* **7**, 101–157

Zaki, M. M. (1965) *Jpn. J. Vet. Res.* **77**, 941