Studies on the Bacteriolytic Activity of *Streptomyces albus*

Culture Filtrates. 2. Fractionation on Ion-exchange Chromatography and Gel Filtration

L. AKSNES and A. GROV

The University of Bergen, School of Medicine, The Gade Institute,
Department of Microbiology, N-5000 Bergen, Norway

Experiments in fractionation of the various lytic activities of *Streptomyces albus* cultures have been carried out using Amberlite IRC-50 and Carboxy methyl cellulose cation-exchangers, electrophoresis in agarose and polyacrylamide gel, and gel filtration on Sephadex. Apparently pure *N*-acetylmuramyl-L-alanine amidase and endo-*N*-acetylmuramidase were isolated on carboxy methyl cellulose columns, whereas all endo-peptidase activities, and one caseinolytic activity were eluted together. Neither on electrophoresis nor gel filtration were the latter activities separated. These results, in addition to optimal pH and salt concentration, indicate that apparently different enzyme specificities had very similar molecular properties.

In a preceding paper¹ the study of strain selection, optimal conditions for enzyme production, and a screening of the enzyme specificities in the culture filtrates of *Streptomyces albus* G were described. Degradation of mucopeptide/cell wall releasing oligosaccharides with the C-1 position of muramic acid free, without detectable free reducing endgroup of glucosamine, showed the presence in the culture filtrates of an endo-*N*-acetylmuramidase. Amidase activity, i.e. *N*-acetylmuramyl-L-alanine amidase, was demonstrated by the release of oligosaccharides free of amino acids, and dinitrophenyl tracing of *N*-terminal amino acids of digests of mucopeptide/cell wall of various bacteria indicated the presence of several endopeptidases. In addition caseinolytic activity was demonstrated.

The present paper comprises the results of the attempts to separate highly purified, specific enzymes from the culture filtrate.

MATERIALS AND METHODS

Chemicals. Amberlite IRC-50 (H⁺), analytical grade, and *N,N'*-methylene-bisacrylamide were obtained from British Drug Houses Ltd., Poole, Dorset, England; Carboxy methyl cellulose (CMC) cation exchanger (lot no. 39B-04701, medium mesh, 0.66 mequiv./g), cytochrome C and β -alanine from Sigma Chemical Co., Mo., U.S.A., and acrylamide from Eastman Organic Chemicals Department, Rochester, N.Y., U.S.A. Acrylic acid, polyethylene glycol (20 000) and 2,4-lutidine were purchased from Fluka AG, CH-9470 Buchs, Switzerland; agarose from Behringwerke AG, Marburg a.d. Lahn, BRD, Sephadex G-75 and blue dextran 2000 from Pharmacia, Uppsala, Sweden, and dialyzer tubing from A. H. Thomas Co., Philadelphia, Pa., U.S.A. Coomassie brilliant blue was obtained from Serva, D-6900 Heidelberg, BRD; *N,N,N',N'*-tetramethylethylenediamine, riboflavin, and ammonium persulphate for disc electrophoresis from Canaco, Rockville, Md., U.S.A., and Freund's complete adjuvant from Difco Laboratories, Detroit, Mich., U.S.A. Hen egg-white lysozyme (3 $\times$ crystalline) was from Calbiochem. Inc., Los Angeles, Calif., U.S.A.; myoglobin from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England, and chymotrypsinogen from Boehringer, Mannheim, BRD. The other chemicals used were as described previously.¹

The organisms, *Staphylococcus aureus* Copenhagen (Sa), *Streptococcus pyogenes* A (Sp), *Planococcus* 2389 (Pc), *Micrococcus luteus* 144 (M-144), *M. conglomeratus* 84 (M-84), *M. lyso-deikticus* NCTC 2665 (MI), *Sarcina lutea* (SI) and

Bacillus megaterium KM (Bm), media, growth and harvesting, mucopeptides/cell wall, thin-layer chromatography, tests for free groups, tests for bacteriolytic and caseinolytic activities were as described or referred to in Ref. 1.

Absorption to and elution from Amberlite IRC-50.¹⁻³ A detailed description of the procedure has been given earlier,¹ and the dialyzed eluate (0.01 M Tris-HCl pH 8.0) is referred to as crude enzyme preparation (CEP). Experiments were also carried out in which washed, enzyme-loaded resin was transferred to columns and eluted with a salt-gradient (0–1 M NaCl) or stepwise with buffers of increasing pH (4.5–9.0). Concentration of enzyme solutions was obtained using polyethylene glycol in dialyzer tubing.

Fractionation on carboxymethyl cellulose.²⁻⁵ CMC was pretreated with 0.2 M NaOH and 0.2 M HCl, washed in 0.05 M Tris-HCl, pH 8.0 to constant pH, equilibrated against 0.01 M Tris-HCl, pH 8.0, evacuated to remove air-bubbles, and then applied to a column (3.6 cm diam. $\times$ 3.0 cm) which was given a hydrostatic pressure of 20 cm. Enzyme solutions were applied to the column at a rate of 10 ml/h, then washed with 500 ml of 0.01 M Tris-HCl pH 8.0, the same buffer being used for elution arranged to give an NaCl-gradient from 0 to 0.2 M in 1 l elution buffer. The elution rate was 20 ml/h and 10 ml fractions were collected.

Gel filtration on Sephadex G-75. Sephadex G-75 in 0.01 M Tris-HCl, pH 8.0, containing 0.02 % Na-azide, was applied to a column (1.8 $\times$ 95 cm) and the void volume determined using blue dextran. After application of 2 ml of material, elution was performed with 0.01 M Tris-HCl pH 8.0 at a rate of 20 ml/h using a peristaltic pump (LKB Produkter, Stockholm, Sweden). Fractions of 5 ml were collected.

Apparent molecular weight was determined by the method of Andrews⁶ using the Sephadex G-75 column described above and cytochrome C, egg-white lysozyme, myoglobin, and chymotrypsinogen as references.

Electrophoresis in agarose was performed according to the method of Scheidegger⁷ using an LKB 6800 A immunoelectrophoretic apparatus (LKB Produkter).

Detection of bacteriolytic activity was attained by sandwiching approx. 0.5 mm of 1 % special agar Noble in 0.01 M Tris-HCl pH 8.0 containing 5 mg/ml of heat-killed bacteria, over the electrophoretic slides followed by incubation at 37°C.

Caseinolytic activity was similarly detected using 0.3 % casein in 1 % agar. After incubation the slides were placed in 5 % trichloroacetic acid to visualize undigested casein.

Polyacrylamide gel (disc) electrophoresis was carried out essentially according to the method of Ornstein⁸ using two running systems: I as described by Broom⁹ and II as described by Joice and Klemperer,¹⁰ both previously used for

separation of basic proteins. Ten μ l of enzyme solutions dialyzed against the electrodic buffer (diluted 1/4)¹¹ and made up to 50 % in glycerol, were applied to the top of the gels. The equipment used was a Shandon disc electrophoresis apparatus (Shandon Scientific Company Ltd., London, England). The staining procedure was as described by Maurer.¹² Detection of bacteriolytic and caseinolytic activities in the gels were principally carried out as described above. The buffer used for gels run in system II was 0.05 M Tris-HCl pH 8.5. The gels were divided along the long axis and placed on agar containing heat-killed bacteria/casein.

Antisera. Rabbits were injected intramuscularly with enzyme solutions, 80 units/ml¹ dialyzed against 0.07 M sodium phosphate, pH 7.2, containing 0.15 M NaCl, mixed with an equal volume of Freund's complete adjuvant. Two injections (1 ml of enzyme solution/dose) were given 3 weeks apart and the animals were bled 2 weeks after the last injection. Sera were heated at 56°C for 30 min to inactivate complement, 1 % merthiolate was added as preservative (one drop/10 ml serum) and the sera were then stored at –25°C.

Serological techniques. Examinations for precipitating antibodies were performed by ring test¹³ and double diffusion in agar.¹⁴ Immunoelectrophoresis was carried out with an LKB apparatus as described by the manufacturer, and inhibition of bacteriolytic activity by serum was tested after incubation at 37°C for 2 h of 1/1 mixtures of serum, diluted in the phosphate-buffered saline, and enzyme in 0.01 M Tris-HCl pH 8.0.

RESULTS

Amberlite. The CEP (dialyzed eluate from the resin) was as previously¹ found to contain the same lytic spectrum as that of the culture filtrates. In spite of a low yield (approximately 50 % of the total bacteriolytic activity of the culture filtrate) the enzymes of large volumes were rapidly isolated in relatively small volumes. At the same time a 100 fold purification was obtained¹ in relation to the absorbance at 280 nm. The fractionation experiments using salt-gradient or stepwise elution with increasing pH were both unsuccessful. The lytic material was in both cases released in a large elution volume without detectable separation of different activities. All further fractionation experiments were carried out on CEP.

CM-cellulose. Some caseinolytic but no bacteriolytic activity was observed in the washings. Every second fraction of the eluate

Fig. 1. Lytic activity in fractions from the CMC-column, eluted with NaCl-gradient, as tested on heat-killed cells of Bm (O), Sa (●), Ml and Sl (■), M-84 and M-144 (▼), and on Sp (---). ΔT_{60} (reduction in turbidity after 60 min) = $(\Delta T_t/t) \times 60$.

Fig. 2. Fractions from the CMC-column, eluted with NaCl-gradient (---), showing caseinolytic activity (●), increase in free amino groups (O) tested on Sa mucopeptide, and increase in reducing groups (■) and *N*-acetylaminosugars (▼) tested on Bm cell wall. ΔT_{60} : See legend Fig. 1.

was tested for bacteriolytic activity on heat-killed bacteria, caseinolytic activity and increase of free groups after digestion of Sa-mucopeptide and Bm-cell wall (Figs. 1 and 2). Two peaks with lytic activity were revealed; one (1) with lytic activity on all the test bacteria, the other (2) only for Sa and Bm when incubation time was 90 min. Longer incubation time showed trace of lysis on the other bacteria as well. Fractions were bulked as indicated by the horizontal lines in Fig. 1. The caseinolytic activity was eluted in one peak (Fig. 2) which overlapped peak 1. Tests on ethylene-oxide treated Sa-mucopeptide and Bm-cell wall showed the same elution profile. Fractions increasing the amount of free amino groups corresponded to peak 1, whereas fractions increasing the amount of reducing groups and of free *N*-acetylaminosugars corresponded to peak 2. The fractions showed no absorbance at 280 nm.

The occurrence and degree of activity of peak 2 varied considerably from one culture to another. When peak 2 did not appear, digestion of mucopeptide/cell wall with culture filtrate showed no increase in reducing groups or *N*-acetylaminosugars. The yield of bacteriolytic and caseinolytic activities was 75–

Fig. 3. Lytic activity in eluate from Sephadex G-75 on heat-killed cells of Bm (●), Sa (■), Ml and Sl (---), M-84, M-144 and Sp (....). Each fraction was 5 ml. ΔT_{60} : See legend Fig. 1.

Fig. 4. Fractions (5 ml) from Sephadex G-75 column showing caseinolytic activity (---), increase in free amino groups (●) tested on Sa mucopeptide, and increase in reducing groups (■) and *N*-acetylamino sugars (···) as tested on Bm cell wall. ΔT_{60} : See legend Fig. 1.

80 % and about 30 %, respectively. Other dimensions of column or changes in the buffer system and salt gradient did not give better separation of different activities or the two peaks.

Sephadex G-75. Figs. 3 and 4 show the result of filtration on Sephadex G-75 of 10 ml CEP which had been concentrated to 2 ml following dialysis against 0.01 M Tris-HCl pH 8.0. All bacteriolytic and caseinolytic activity was eluted in one peak, the fractions of which also increased the amount of free amino groups, reducing groups and *N*-acetylamino sugars upon digestion of ethylene-oxide treated Sa-mucopeptide and Bm-cell wall. No absorbance

was detected at 280 nm. Apparent molecular weight of the material in the peak was calculated to be 16 000.

Maximal release of free groups by various enzyme fractions. The increase in the amount of free amino groups, reducing groups, and *N*-acetylamino sugars of ethylene-oxide treated mucopeptide/cell wall after digestion with CEP, CMC-peak 1 and CMC-peak 2, is given in Table 1. All enzyme solutions were concentrated to about 30 units/ml and dialyzed before incubation with substrate until constant values were observed. All the three enzyme solutions gave a 100 % reduction in turbidity of all mucopeptide/cell wall preparations. In comparison egg-white lysozyme gave about 80 % reduction in turbidity under the conditions employed. It appears from Table 1 that there is a general increase in free amino groups in all digests, but that the effect of the CEP is markedly higher than that of the two other enzyme solutions. The CEP and CMC-peak 2 showed a significant increase in the amount of reducing groups and *N*-acetylamino sugars, whereas only a very small increase was observed in these after treatment with CMC-peak 1.

Specific activities. Dinitrophenyl-tracing of ethylene-oxide treated mucopeptide/cell wall digested with CMC-peak 1 revealed a significant increase of DNP-alanine only in M-84 (Table 2). The digests of M-144 and M1 mucopeptides contained DNP- ϵ -lysine, Sa mucopeptide DNP-glycine, Pc mucopeptide DNP-glutamic acid and Bm cell wall contained DNP-diaminopimelic acid. This indicates the presence of the same peptidase activities as demonstrated in culture filtrates,¹ whereas the amidase seems to be absent. Reduction with NaBH_4 , hydrolysis

Table 1. Maximal increase ($\mu\text{mol/mg}$) of free amino groups (DNP), reducing groups (P-J) and *N*-acetylamino sugars (M-E) after digestion of mucopeptide/cell wall with crude enzyme preparation (CEP), CMC-peak 1 and CMC-peak 2.

Substrate	CEP			CMC-peak 1			CMC-peak 2		
	DNP	P-J	M-E	DNP	P-J	M-E	DNP	P-J	M-E
M-84 mucopeptide	400	200	85	240	20	0	150	200	80
M-144 »	120	90	40	80	0	0	70	80	30
M1 »	110	80	20	75	0	0	70	80	25
Sa »	450	220	90	260	30	0	200	210	85
Pc »	310	150	70	210	35	0	150	145	75
Bm cell wall	200	230	100	50	25	0	35	210	100

Table 2. Dinitrophenyl(DNP)-amino acids detected chromatographically after treatment of mucopeptides/cell wall with CMC-peak 1.

	Mucopeptide ^a		MI	Sa	Pc	Cell wall ^a Bm
	M-84	M-144				
DNP-Ala	+++	—	—	(+)	+	—
DNP-Gly	—	—	—	+++	—	—
DNP-Glu A	—	—	—	—	+++	—
DNP-ε-Lys	—	+++	+++	—	—	—
DNP-DAP	—	—	—	—	—	++

^a (+) to +++: weak to strong colour reaction.

and chromatography of mucopeptide digested with CMC-peak 2 revealed appearance of muraminitol and disappearance of muramic acid, contrary to the findings with non-reduced digest. This shows that the muramidase, previously demonstrated in the culture filtrate,¹ is separated from the major portion of peptidases on CMC and located to peak 2. Search for the amidase activity observed in the culture filtrate¹ showed that, under the conditions described, the amidase was not bound to the CMC-column and could be obtained from the effluent.

Electrophoresis in agarose. Samples of culture filtrate, CEP, and CMC-peak 1, all concentrated to approximately 40 units/ml, were subjected to electrophoresis in agarose at pH 8.6 and 250 V for 120 min. Lytic activity was tested on all test bacteria and casein, a representative picture being shown in Fig. 5. The bacteriolytic activity seemed electrophoretically homogeneous, showing activity on all test bacteria in the same

Fig. 5. Electrophoresis in agarose, pH 8.6, 250 V, 120 min, of CEP (1) and CMC-peak 1 (2) tested for caseinolytic (A) and bacteriolytic activity (B).

spot, and identical mobility of this activity of both culture filtrate, CEP, and CMC-peak 1. Several spots with caseinolytic activity were revealed in CEP (Fig. 5A) and culture filtrate, whereas only one caseinolytic spot, having identical mobility with that of bacteriolytic activity, was demonstrated with CMC-peak 1.

Disc electrophoresis. Samples of CEP, CMC-peak 1, and a 1/1 mixture of CMC-peaks 1 and 2, all concentrated to approximately 40 units/ml and dialyzed, were run at pH 8.3 and pH 4.3. For all enzyme solutions only one active area, having lytic activity on all test bacteria and casein, was observed. Fig. 6 shows the position

Fig. 6. Mobility of caseinolytic (A) and bacteriolytic (B) activity on (I) disc electrophoresis in system I (1 ma/tube for 10 min + 3 ma/tube for 50 min) and (II) system II (50 V for 10 min + 150 V for 50 min). Egg-white lysozyme (C) was included for comparison and visualized by staining. In system I, lysozyme was first run for 10 min with 1 ma/tube then for 25 min with 3 ma/tube.

of caseinolytic activity (A), bacteriolytic activity (B) and stained lysozyme (C) after electrophoresis in systems I and II.

Immunology. In contrast to the preimmune sera, rabbit antisera prepared against CEP and

CMC-peak 1 contained precipitating antibodies against the enzyme solutions. Double diffusion in agar using undiluted sera and enzyme solutions concentrated to about 40 units/ml, showed reaction of identity between CEP and CMC-peak 1 with only one detectable precipitin line. A comparison between bacteriolytic activity (Fig. 7A) and serological activity (Fig. 7B) after immunoelectrophoresis (250 V, 120

Fig. 7. Immunoelectrophoresis (250 V for 120 min) of CEP (1) and CMC-peak 1 (2) following tests for bacteriolytic (A) and serological (B) activities.

min) of CEP and CMC-peak 1 (40 units/ml), showed that the electrophoretic movement of the antigen responsible for the precipitin line was less than that of lytic activity. Experiments in inhibition of lytic activity by sera showed that both immune sera and preimmune sera gave complete inhibition, the immune sera being most effective, *i.e.* inhibiting at the highest dilution.

Kinetics. Measurements of the decrease in turbidity at 600 nm of suspensions of heat-killed bacteria and enzyme solutions as a function of time showed a linearity for all enzyme preparations and all test bacteria until approximately 65 % reduction. Thereafter the curves became flatter.

Serial dilutions (in 0.01 M Tris-HCl pH 8.0) of the enzyme preparations all showed the same relative reduction in activity on all test bacteria. Fig. 8 illustrates the results of CEP tested on heat-killed Sa and Bm cells.

The influence of variation in pH on the lytic activity of CEP and CMC-peak 1 was tested between pH 3.5 and 9.5 using Na-

Fig. 8. Lytic activity of dilutions of CEP on heat-killed Bm (O) and Sa (●) bacteria. ΔT_{60} : See legend Fig. 1.

acetate (pH 3.5–5.5), Na-phosphate (pH 5.5–7.5) and Tris-HCl (pH 7.5–9.5) buffers. Heat-killed bacteria (1 mg/ml of water), buffer (0.04 M) and enzyme solution (dialyzed against 5 mM Tris-HCl pH 7.5) were mixed at a ratio of 2:3:1. All enzyme solutions showed maximal activity on all test bacteria at a pH of approximately 8.5.

Lytic activity on heat-killed bacteria of both CEP and CMC-peak 1 in 0.01 M Tris-HCl pH 8.0, showed maximum at a NaCl concentration of approximately 0.01 M. Higher concentrations of NaCl inhibited the lytic activity, which was nearly zero at 0.16 M NaCl. The same conditions, pH 8.5 and 0.01 M NaCl, were also found to be optimal for digestion of mucopeptides.

The bacteriolytic activity of CEP or CMC-peak 1, tested on all bacteria in 0.01 M Tris-HCl pH 8.0, was not influenced by the presence of casein (0.5 mg/ml).

Stability. Enzyme solutions stored at 4°C in 0.01 M Tris-HCl pH 8.0, 0.02 % Na-azide, showed a high degree of stability. After one month about 5 % loss in lytic activity was observed. The enzymatic activities were also stable at –25°C.

DISCUSSION

The crude enzyme preparation (CEP) used in the present fractionation experiments corre-

sponds to the materials in other studies of *Streptomyces albus* G.²⁻⁵ The bacteriolytic activity observed was completely bound to CM-cellulose, and fractionation on columns usually gave two peaks with lytic activity (Fig. 1). The material of peak 1, starting elution at 0.02 M NaCl and thus being weakly bound, contained practically all the endopeptidase activities. Digestion of mucopeptides/cell wall increased the amount of free amino groups, and the ratio between the degree of activity on the different test bacteria was approximately the same as that of CEP. Removal of the activity in CMC-peak 2 thus seemed to have little influence on the total lytic activity. Under the conditions imposed, peak 2 lysed mainly *B. megaterium* and *S. aureus*, and varied quantitatively from one culture to another. The material (peak 2) lysed mucopeptides/cell wall and increased the amount of free reducing groups and *N*-acetyl-amino sugars, whereas the increase of free amino groups was very small, the accessibility of these groups for reaction with FDNB probably being due to solubilization. This indicates that the activity of peak 2 is mostly dominated by an endo-*N*-acetylhexosaminidase which is shown to be an endo-*N*-acetylmuramidase. The properties of this agree well with those of the F₁-endo-*N*-acetylmuramidase⁴ which also showed a somewhat stronger adsorption to CM-cellulose than the endopeptidases.

Optimal pH of the F₁-enzyme for lysis of different bacteria has been found to vary considerably (pH 3.5–8.0).⁴ This may explain the difference in lytic activity and in the release of reducing groups on testing different bacteria with fractions of CMC-peak 2 and other solutions supposed to contain muramidase activity.

Mixtures of certain lytic activities from *Streptomyces albus* G have been found to act synergistically,^{15,16} and the activity peaks of eluates from CMC-columns may therefore be due to favourable mixtures of enzymes and not to real quantities. Due to the low protein content indicated spectrophotometrically (280 nm), this was difficult to determine.

The *N*-acetylmuramyl-L-alanine amidase demonstrated in the CEP¹ was not adsorbed to CM-cellulose under the conditions imposed. This was also found in earlier studies.^{2,3} The non-adsorbed material, containing the amidase, showed no lytic activity, thus suggesting, in

agreement with an earlier supposition,¹⁷ that the amidase is non-lytic on whole bacteria.

Electrophoresis in agarose and polyacrylamide and also gel filtration on Sephadex showed only one zone of bacteriolytic activity, indicating that the molecular properties of the apparent different enzyme specificities are very similar. Separation of several activities of *Streptomyces* culture filtrates on CMC-columns has been reported.²⁻⁵ However, although all endo-peptidases reported, including MR, SA, ML, and KM, also seemed to be present in the CEP,¹ no separation was obtained.

Several caseinolytic enzymes were present in the CEP and could be separated on electrophoresis in agarose and by adsorption to CMC. The caseinolytic activity bound to CMC was eluted together with bacteriolytic activity in peak 1, these activities being also associated after electrophoresis and gel filtration. This suggests the possibility that the same enzyme might have bacteriolytic and caseinolytic activity, but casein in the test-solution did not seem to influence the degree of bacteriolysis. Caseinolytic activity has previously been found in association with the MR endo-peptidase.^{2,3}

Most bacteriolytic enzymes isolated have been found to be basic proteins with a high isoelectric point.^{2,3,18} The results of electrophoresis in gel in the present experiments also indicated basic proteins with pI-values above 8.6. According to the apparent molecular weight (16 000) it is reasonable to expect that the observed difference between the lytic enzymes and egg-white lysozyme (pI 11.0, Mw 14 000)¹⁹ in electrophoretic mobility on polyacrylamide gel (Fig. 6) is due to difference in charge. On the other hand, due to the basic character of the enzymes, a possible retardation on Sephadex which influences the molecular weight determination,²⁰ cannot be excluded.

With a few exceptions,^{4,21} all lytic enzymes previously isolated from *Streptomyces albus* G have been shown to have pH optima in the same range as found in this study,^{15,16,22} and the low optimal salt-concentration is also in accordance with earlier findings.

The immunoelectrophoretic experiment showed that the material responsible for precipitation with antiserum in agar was different from that with lytic activities. However, this antigenic material does not need to be present

in high amounts since the ability of proteins to evoke immunological response may vary considerably.²³ Although the sera showed high activity of non-specific inhibitors,^{24,25} the inhibition studies may suggest that the antisera contained specific inhibiting antibodies.

The reported results of fractionation studies on the lytic activities of *Streptomyces* cultures show great diversity, to some degree probably due to treatment and selection of the strain for enzyme production. In the present experiments fractionation on CMC-columns separated the CEP into three portions: (1) the material not adsorbed, containing non-lytic *N*-acetylmuramyl-L-alanine amidase, (2) CMC-peak 1 containing endo-peptidases, free of detectable amidase and hexosaminidase activities, and (3) CMC-peak 2, consisting mainly of endo-*N*-acetylmuramidase.

REFERENCES

1. Aksnes, L. and Grov, A. *Acta Chem. Scand. B* 28 (1974) 185.
2. Petit, J. F., Munoz, E. and Ghuysen, J.-M. *Biochemistry* 5 (1966) 2764.
3. Ghuysen, J.-M., Dierickx, L., Coyette, J., Leyh-Bouille, M., Guinaud, M. and Campbell, J. N. *Biochemistry* 8 (1969) 213.
4. Munoz, E., Ghuysen, J.-M., Leyh-Bouille, M., Petit, J. F. and Tinelli, R. *Biochemistry* 5 (1966) 3091.
5. Ghuysen, J.-M., Leyh-Bouille, M., Bonaly, R., Nieto, M., Perkins, H. R., Schleifer, K. H. and Kandler, O. *Biochemistry* 9 (1970) 2955.
6. Andrews, P. *Biochem. J.* 91 (1964) 222.
7. Scheidegger, J. J. *Int. Arch. Allergy Appl. Immunol.* 7 (1955) 103.
8. Ornstein, L. *Ann. N. Y. Acad. Sci.* 121 (1964) 321.
9. Broom, J. *Nature (London)* 199 (1963) 179.
10. Joice, J. R. and Klemperer, H. G. *Anal. Biochem.* 41 (1971) 581.
11. Hjertén, S., Jerstedt, S. and Tiselius, A. *Anal. Biochem.* 11 (1965) 219.
12. Maurer, H. R. *Disc-electrophoresis*, Walter de Gruyter, Berlin 1968.
13. Kabat, E. A. and Mayer, M. M. *Experimental Immunochemistry*, Thomas, Ill., U.S.A. 1964.
14. Ouchterlony, Ö. *Acta Pathol. Microbiol. Scand.* 26 (1949) 507.
15. Ghuysen, J.-M. *Arch. Int. Physiol.* 65 (1957) 173.
16. Salton, M. R. J. and Ghuysen, J.-M. *Biochim. Biophys. Acta* 24 (1957) 160.
17. Ghuysen, J.-M. *Bacteriol. Rev.* 32 (1968) 425.
18. Wadström, T. and Vesterberg, O. *Acta Pathol. Microbiol. Scand. Sect. B.* 79 (1971) 248.
19. Young, E. G. *Compr. Biochem.* 7 (1963) 25.
20. Andrews, P. *Methods Biochem. Anal.* 18 (1970) 1.
21. Ghuysen, J.-M. and Strominger, J. L. *Biochemistry* 2 (1963) 1100.
22. Ghuysen, J.-M., Dierickx, L., Leyh-Bouille, M., Strominger, J. L., Bricas, E. and Nicot, C. *Biochemistry* 4 (1965) 2237.
23. Sela, M. *Ann. N. Y. Acad. Sci.* 190 (1971) 181.
24. Arnon, R. and Neurath, H. *Proc. Natl. Acad. Sci. U.S.A.* 64 (1969) 1323.
25. Fossum, K. *Acta Pathol. Microbiol. Scand. Sect. B.* 78 (1970) 605.

Received October 13, 1973.