THE PHAGE RECEPTORS OF BACILLUS ANTHRACIS

By

JUDITH LANTOS and G. IVÁNOVICS

Institute of Microbiology, University Medical School, Szeged

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Summary. In the cell wall of *Bacillus anthracis* the presence of distinct receptors specific for certain groups of A phages has been demonstrated.

Individual receptors are bound to different structural elements of the cell wall. The receptors could not be identified with the immune-specific polysaccharide of *B. anthracis*.

The immune-specific polysaccharide, a galactose-glucosamine-polymeric peptide, is a characteristic component of the cell wall which cannot be isolated by mild chemical treatment but goes into solution on enzymatic autolysis.

We have recently reported about the isolation of phages from soil samples [1, 2], which had specific lytic activity on *B. anthracis*. Some representatives of these phages were extensively studied. For the designation of this group of agents the name "A phage" was suggested. According to their sensitivity to heat and UV irradiation, furthermore to some other characteristics, especially their antigenic structure, the A phages could be divided into 3 groups [2]. In this classification the α-mutant of phage W isolated by McCLOY [3] from a lysogenic strain of *B. cereus* was used as reference agent. Group Al was represented by phage W which has been known to be highly specific for *B. anthracis*. A sharp difference was found in the antigenic structure of phages belonging to groups Al and A3, while certain serologic relationships were found between group Al and A2.

The mutants of *E. coli* resistant to T phages exhibit a remarkable host specificity which may serve as a basis for a differentiation of the T system. To allow a more thorough differentiation of A phages, it was attempted to isolate hosts which are resistant to individual A phages. Except for phage W this attempt has failed. Thus resistant mutants of *B. anthracis* which determine the specificity of receptors for individual groups of phage were only available for Al phage. The characterization of the phages was also attempted by the isolation of A-phage receptors. This attempt has also failed as we did not succeed in solubilisation of the receptors specific for A phages. In spite of the failure some observations have been made which might prove valuable for a further characterization of A phages. The present paper will deal with these observations.

Materials and methods

Bacterial strains. Most of our experiments were performed with an atypical asporogenous *B. anthracis* strain Davis. A non-capsulogenic mutant of the Vollum strain (VC⁻) was also used. The non-capsulogenic strain resistant to W*a* phage was isolated in connection with the lysogenization experiments carried out with the Vollum strain [4].

Cultivation of bacteria. It was carried out in a fluid medium under constant shaking in YP bouillon as described earlier. Growth and cell count of the cultures were determined by measuring their optical density, as described previously [2].

Phage strains were described and characterized in a previous paper [2]. The groups and the trains of phages were:

Phage A1,	strain W*a*
Phage A2,	strains 26 *cr* and 27 *cr*
Phage A3,	strains 26 *cs*, 26 *tl*, 27 *tl*, 28 *tl*,*cg*, 29 *cg*.

The phage material was produced on strain Davis cultivated in fluid medium. The lysates were centrifuged and sterilised by shaking with chloroform for a few minutes. Titres of the lysates were 10^9-10^{10}/ml.

Phage assays were made according to ADAMS [5].

Preparation of bacterial suspensions for receptor studies. Cell suspensions were usually prepared from 16-hour cultures of strain Davis and sometimes from those of strain Vollum (VC⁻), both cultivated on YP agar. The cells were suspended in chilled saline and washed twice by centrifugation in the cold. The sediment was re-suspended in saline and diluted to contain 20 mg/ml dry weight of bacteria, as determined by optical density.

Cell-wall preparations. Cell-wall preparations were obtained from washed suspensions as described by PARK and HANCOCK [6]. Fifty ml of the bacterium suspension was centrifuged and re-suspended in 20 ml of 5 per cent trichloroacetic acid (TCA) at 4° C. The material was allowed to stand for 30 minutes at this temperature. This was followed by centrifugation and the sediment was washed in 75 per cent ethanol, re-suspended in 5 per cent TCA and heated to 90° C for 6 minutes. After centrifugation the sedimented bacteria were washed in water and re-suspended in 0.05 *M* ammonium chloride solution. The pH of the latter was adjusted to pH 7.6 by NH_4 OH. After addition of 1 mg/ml of crystalline trypsin the material was digested for 2 to 3 hours at 37° C.

The process was followed by examination under a phase-contrast microscope. If the treatment did not result in removing the cell content, trypsin digestion was repeated. Digestion was followed by washing in water three times. The final suspension was stored in a refrigerator.

Even after successful trypsinisation it was possible to identify inside the cell wall a shrunk dark central part, apparently a residue of the cytoplasmic membrane. This mass was remarkably electron dense, as demonstrated in Fig. 1. It could not be removed by solvents or enzymes (lipase, lysozyme, chymotrypsin, pepsin, ribonuclease). Extraction of the cell-wall preparations with 90 per cent phenol resulted in further shrinkage of the electron dense body, and in further increase of its density (Fig. 2.). The electron micrographs revealed wrinkling of the cell wall at the poles.

Determination of the receptor substance. The quantity of receptor substance in bacterial suspensions and cell-wall preparations was estimated on the basis of their phage adsorbing capacity. Living, appropriately treated bacteria or cell-wall preparations were suspended in YP broth to a density of 5×10^7 cells or cell walls per ml. An equal amount of YP broth containing 10^7 phage particles was added to the suspensions (multiplicity 0.2), the mixtures were allowed to stand at 37° C for 30 minutes, then diluted 1 : 100 by cold diluent (one volume YP broth and 4 volumes saline). The diluted suspensions were centrifuged at 3000 rpm for 10 minutes. The supernatants were again diluted 1 : 100, and 0.1 ml of this dilution mixed with indicator organisms was overlayered in soft agar.

The receptor content of cell-free extracts was determined by their phage-inactivating activity. As described by WEIDEL [7], this reaction is pseudomonomolecular and irreversible in the presence of an excess of dissolved receptor. To secure an appropriate receptor excess, phage was added to preparations corresponding 5×10^7/ml bacteria in 0.001 multiplicity.

Chemical examination of bacterial and cell-wall preparations. Nitrogen content was determined by nesslerization according to MILLER and MILLER [8]. The method of FISKE and SUBBAROW [9] was used for phosphorous determination. Anthrone-positive materials were determined as suggested by SEIFTER *et al.* [10].

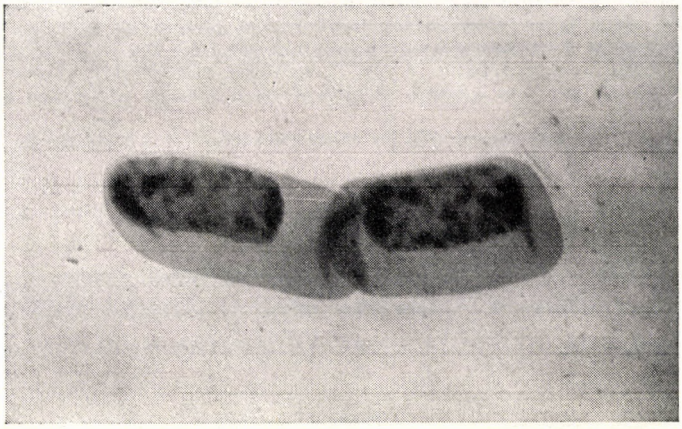

Fig. 1. Cell-membrane preparation from strain Davis (according to PARK and HANCOCK) Magnification × 10 000

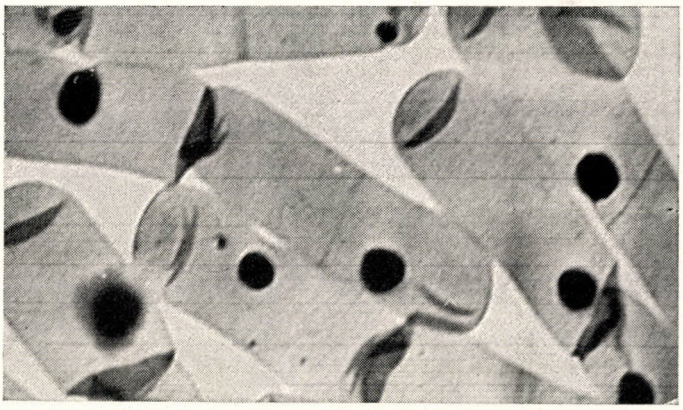

Fig. 2. Cell-membrane preparation after phenol extraction. Magnification × 18 000

For the examination of the cell-wall components, samples containing 20 mg of dry substance were mixed with 1 ml 2*N* HCl, sealed in ampoules, and kept at 100° C for 2 hours. The bulk of hydrochloric acid was removed by distillation under reduced pressure, while the residual acid was removed in an NaOH exsiccator.

The hydrolysate thus obtained was used for the determination of the total reducing substance by the method of HAGEDORN and JENSEN [11]. The results obtained were expressed in glucose values. The amount of hexosamine present was determined according to NEUHANS and LETZRING [12]. The individual monosaccharide components of the hydrolysates were determined by one dimensional ascending paper chromatography, on Whatman's paper No. 1. Two solvents were used in parallel, (a) butanol-pyridine-water (6 : 4 : 3), and (b) pyridine--ethyl-acetate-water (7 : 10 : 3). The chromatograms were developed either by aniline phosphate or by ammonium silver nitrate solution.

For the analysis of the amino-acid component the preparations were hydrolysed by 6 *N* HCl at 105° C for 8 hours. After having removed the acid, 20 μg amounts were used for paper chromatography. Two dimensional chromatograms were run in pyridine-water (4 : 1), and N-butanol-acetic acid and water (6 : 1 : 2) solvents, respectively. The chromatograms were developed by 0.1 per cent solution of ninhydrine in acetone and dryed at 105° C for 5 minutes.

The lipid content was determined by ether extraction of a hydrolysate in 2*N* HCl.

Results

The sensitivity of VC⁻/a, a Wa phage resistant strain, to heterologous phages. Various dilutions of phages were dropped onto the surface of indicator plates prepared with strains VC⁻ and VC⁻/a. The results are presented in Table I.

Table I

Titre of different phages on sensitive VC⁻ and resistant VC⁻/a strain

Phage group	Phage strain	Phage titre (logarithmic value of dilution)	
		VC⁻	VC⁻/a
A1	Wa	7	0*
A2	*26 cr*	7	5
	27 cr	6	6
A3	*26 cs*	6	5
	26 tl	7	7
	27 tl	7	6
	28 cs	7	6
	28 tlcg	6	6
	29 cg	7	6

* Plane lysate had no effect.

As revealed by Table I strain VC⁻/a resistant to phage Wa was just as sensitive to heterologous phages as the original strain VC⁻. In adsorption experiments strain VC⁻/a failed to adsorb phage Wa, while no changes could be observed in the adsorption of some representants of the other phage types. We did not succeed in obtaining mutants resistant to other phages, thus no cross experiments could be performed.

Attempts to solubilize the receptor substance. Suspensions of *B. anthracis* were extracted under different conditions and the phage-binding capacity of the bacteria and the phage-inactivating activity of the extracts was estimated. Usually phage Wa was applied, while in some experiments certain representants of groups A2 and A3 (27 *cr* and 29 *cg* strains) were also employed to detect the presence of the receptor.

Samples of the moist bacterial suspension were treated for 30 minutes at room temperature with 0.1 *N* NaOH, 90 per cent phenol, ethylene glycol or glacial acetic acid. None of these treatments had any effect on the phage-adsorbing capacity of the bacteria. Similarly, no decrease in the adsorbing capacity was found on treatment with different enzymes at pH 7 in phosphate buffer. Crystalline trypsin, chymotrypsin, ribonuclease, lysozyme and a lipase concentrate proved equally ineffective.

Washed bacteria were autolysed in the presence of trypaflavine (20 μg/ml) in pH 8 phosphate buffer (*M* 0.02) under a toluene layer at 37°C for 48 hours. The Gram negative cell debris thus obtained was sedimented at 3000 rpm for 10 minutes. The slightly opalescent supernatant had a remarkable inactivating effect on phage Wα, while against the other two it was ineffective. This supernatant, after 30 minutes' centrifugation at 10 000 rpm yielded a little sediment and a clear supernatant. The latter was deprived of inactivating activity, thus apparently the sedimentable bacterial detritus was responsible for the inactivation of phage Wα.

The cell wall of *B. anthracis* could be lysed only by relatively rough methods, e. g. by alkaline NaOCl solution [13] which, however, does not seem to leave intact the original chemical structures. Extraction of the receptor by formamide solution was attempted under different conditions. Lyophilised bacteria were suspended in concentrated formamide and heated. This was followed by repeated washing and testing for phage-binding capacity. No decrease in the adsorbing capacity for phage A3 took place, even after extraction at 170°C for 30 minutes. This procedure, however, remarkably reduced the adsorbing capacity for group A1 and A2 phages. Nevertheless, no active solubilised receptors were demonstrable in the formamide supernatant. Bacteria extracted with formamide were found to have undamaged contours in preparations stained according to GUTSTEIN. The extract contained only traces of the immune specific polysaccharide of *B. anthracis* [14]. Thus even extensive formamide treatment did not result in remarkable solubilization of the cell-wall component. The specific receptors of phages A1 and A2 on the other hand were inactivated on formamide treatment at 170°C.

An 0.1 per cent solution of the immune specific polysaccharide of *B. anthracis* was definitely ineffective against all the phages tested. Thus the receptor substances could not be identified with the galactose-acetyl-glucosamine mucopeptide.

We did not succeed in solubilising the receptors by lysozyme treatment though it is well known that this enzyme is able to lyse completely the bacteria under appropriate experimental conditions. Young cultures grown in YP broth (0.5 o. d.) were centrifuged, the sediment was washed and resuspended in a pH 8 tris-buffer, and 50 μg/ml of lysozyme was added. This treatment resulted in a remarkable decrease of the optical density of the suspension

and no intact bacteria could be detected by microscopic examination. The clear supernatant obtained on centrifugation was added to equal amounts of phages in YP broth and the mixture was assayed for phage at intervals. The extract was found to be ineffective, in inactivating phage; this indicated the absence of active receptor substance in the lysozyme extract.

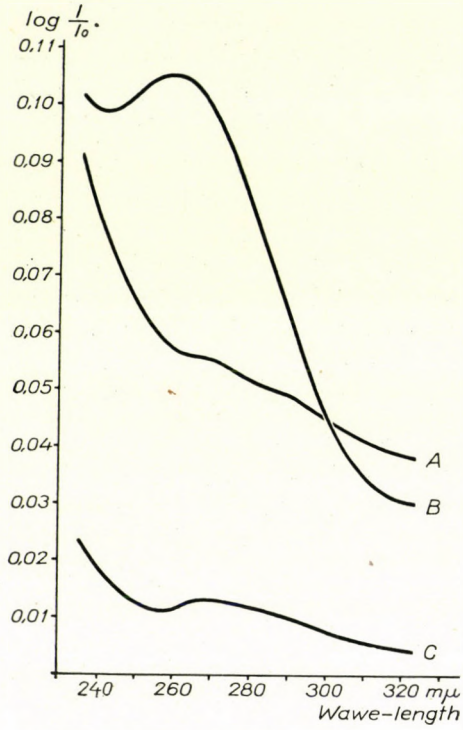

Fig. 3. Changes in the optical density of Davis suspension on different treatments. *A* live untreated suspension; *B* after boiling TCA extraction; *C* trypsin-digested suspension after TCA treatment

Examination of the phage-binding capacity of cell-wall preparations. Fig. 3 demonstrates the UV adsorption of a suspension obtained from strain Davis by extraction with boiling trichloroacetic acid and trypsin digestion.

Extraction with boiling TCA resulted in a remarkable drop of the extinction values in the range between 255 and 266 mμ. The very low adsorption observed for the cell-wall preparations obtained by trypsin digestion revealed a slight inflection at 260 to 280 mμ. Fig. 4 contains the data on the phage adsorption (A1, A2 and A3 group) of the cell-wall preparations at the different phases of PARK and HANCOCK's procedure.

Living, not washed bacterial suspension adsorbed all the 3 phages to 90 to 98 per cent. The rate of adsorption of A1 (W*a*) and A2 *(27 cr)* phages

decreased remarkably during the consecutive treatment, while the adsorbtion of A3 *(29 cg)* was essentially unchanged. The difference in adsorption of individual phages appeared after treatment with boiling TCA, and increased, on further treatment with trypsin and lysozyme.

The experiment demonstrated above was performed with one representative of each phage group. Similar results were obtained in another experiment where all the phage strains belogning to the 3 phage groups were examined.

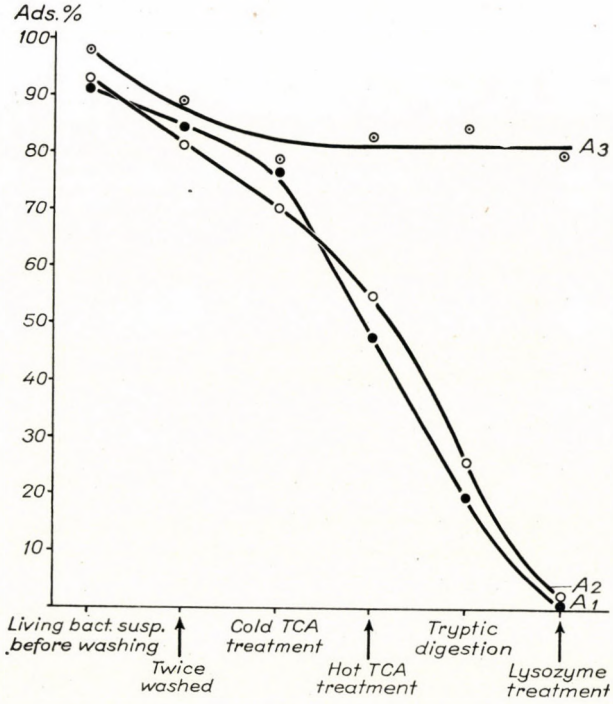

Fig. 4. Phage binding of a suspension of strain Davis (per cent) after different treatments

In this experiment the suspensions of strain Davis were examined for phage adsorption following different treatments.

Short heat treatment caused a moderate decrease in the adsorption of all the phages. Extraction with boiling TCA and trypsin treatment ("cell-wall") resulted in a remarkable decrease in the adsorption of Wα and A2 phages. The same treatment was, however, ineffective on the receptor of phages belonging to group A3, while this receptor was mostly damaged at autolysis. A short heat treatment of bacteria suspended in saline resulted in a slight decrease in adsorption of group A3. Nevertheless this receptor appeared to be resistant to boiling formamide or boiling TCA.

Table II

Phage binding of the suspension of B. anthracis strain Davis after different treatments

Suspension	Grade of adsorption in per cent								
	Members of the individual phage groups								
	A1	A2		A3					
	Wα	26 cr	27 cr	26 cs	26 tl	27 tl	28 cs	28tlcg	29 cg
Live washed bacteria (1)	97	88	90	80	94	91	94	90	98
Heat treated bacteria (2)	88	88	70	70	92	73	83	62	70
Cell wall[636] (3)	10	51	44	85	98	96	92	89	97
Autolysed bacterium (4)	72	46	52	43	8	0	28	23	41

(1) Live Davis suspension.

(2) Five ml of Davis suspension in test tube, kept for 1 minute in a 100° C water bath and cooled at room temperature.

(3) After hot trichloroacetic acid and trypsin treatment according to PARK and HANCOCK.

(4) The suspension was autolysed in the presence of 20 μg/ml trypaflavine in a pH 8 phosphate buffer at 37° C for 48 hours and the phage adsorption of the centrifuged sediment was determined.

Chemical analysis of the cell-wall preparations. Some chemical characteristics of live bacteria of strain Davis and those of different preparations obtained from strain Davis are given in Table III.

Table III

*Some chemical characteristics of washed Davis suspension and cell-wall preparation, calculated according to dry material content**

Suspension No	Nitrogen		Phosphorus		Sugar (anthron)		Reducing material		Hexosamine		Lipid	
	Per cent											
Davis I	9.3		1.2		3.8		4.0		1.8		2.2	
Davis II	11.0	10.7	1.5	1.4	4.8	4.0	4.4		2.3	2.1	3.4	2.8
Davis III	12.0		1.6		3.5		4.3		2.2		2.8	
Cell-wall I	3.5		0.5		8.0		13.6		7.2		2.9	
Cell-wall II	3.6	3.6	0.8	0.6	8.6	7.6	13.4	13.5	7.9	7.2	2.7	3.0
Cell-wall III	3.7		0.6		6.8		13.7		6.6		3.4	

* Results and means of 3 different examinations.

The obtained cell-wall preparations amounted on the average to 25 per cent of the bacterial weight. Only about half of the N and P content of the original bacterial cells was present in the cell-wall preparations. This is in accordance with the decrease in the UV adsorption of the cell-wall

suspension, indicative of a marked decrease in nucleic acid and protein. The carbohydrate content of the preparations appeared to be comparatively higher than that of the bacterial suspension, as measured by the total amount of anthrone-positive and reducing substance. The carbohydrate content of the cell-wall preparations as calculated from the anthrone-positive substances and hexosamine was found to be about 15 per cent. It should be stressed that the hexosamines are anthrone-negative substances. The total amount of reducing substances as determined by the method of HAGEDORN and JENSEN and expressed in glucose gave somewhat lower values. This might be explained by differences in the reducing value of individual carbohydrate components.

Paper chromatography of the cell-wall preparations revealed the presence of galactose and glucosamine. This is in agreement with the chemical composition of the polysaccharide isolated from *B. anthracis* [14]. In certain hydrolysates a faint spot was found of higher R_f than those of glucosamine and galactose. This spot could not be identified. It might have been muramic acid as a faint ninhydrine-positive spot could also be found in the same area. The presence of alanine, glutamic acid, glycine, diamino-pimelic acid and aspartic acid could be demonstrated in hydrolysates of cell wall.

Discussion

Our experiments have yielded certain data concerning the different receptors specific for the different groups of A phages. A good agreement could be observed between the differences in the receptors and the grouping of the A phages based on their antigenic structure and other characteristics (UV, heat and citrate sensitivity). These results have justified our earlier proposal to divide phages A into at least 3 groups.

A mutant of *B. anthracis* resistant to phage W*a* (A1) exhibited unaltered sensitivity to phages A2 and A3. This phenomenon in itself proved the distinctness of the receptors of phage A1. The receptors of phages A2 and A3 can be differentiated on the basis of their behaviour on treatment with hot (170°C) formamide. On autolysing the cells, the receptors of phages A1 and A2 were present in remarkable amounts in the cell debris, while similarly obtained debris were practically ineffective in adsorbing phage A3. This, however, did not mean that the glucose-galactose polymer solubilised on autolysis had any receptor effect on A3 phages.

The cell-wall preparations obtained according to PARK and HANCOCK exhibited an undamaged receptor activity only against A3 phages. Trichloroacetic acid and trypsin treatment was found remarkably to inactivate the other two receptors. On further treatment with lysozyme the A1 receptor was totally destroyed. Extraction with boiling trichloroacetic acid has been known to solubilize ribityl phosphatides (theicoic acid) [6]. This substance,

however, could not be identified with either of the receptors. On the basis of our experiments we may therefore state that the localisation of A1 and A2 receptors is different from that of the A1 receptor.

The immune-specific polysaccharide which is a glucose and galactose polymer containing a peptide moiety [14] was found to be only a part of the structure of the cell-wall preparations obtained from *B. anthracis* by the method of PARK and HANCOCK. The structure of the cell wall of *B. anthracis* appears to be highly resistant to different chemical treatments. The mucopeptide, a structural element of the cell wall, could not be solubilised by any of the chemical methods applied. Enzymatic treatment (lysozyme, autolysis), however, readily dissolved this material from the cell wall.

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LITERATURE

1. IVÁNOVICS, G., LANTOS, J.: Acta microbiol. hung., **5**, 405 (1958).
2. LANTOS, J., VARGA, I., IVÁNOVICS, G.: Acta microbiol. hung., **7**, 31 (1960).
3. MCCLOY, E. W.: J. gen. Microbiol., **18**, 198 (1958).
4. IVÁNOVICS, G.: to be published.
5. ADAMS, M. H.: Methods in Medical Research. The Year Book Publishers, Chicago 1953, Vol. **2**, pp. 1—62.
6. PARK, J. T., HANCOCK, R.: J. gen. Microbiol., **22**, 249 (1960).
7. WEIDEL, W., KOCH, G., BOBOSCH, K.: Z. Naturforsch., 9b, 573 (1954).
8. MILLER, G. L., MILLER, E. E.: Anal. Chem., **20**, 481 (1948).
9. FISKE, C. H., SUBBAROW, Y.: J. biol. Chem., **66**, 375 (1925).
10. SEIFTER, S., DAYTON, S., NOVIC, B., MUNTWYLER, E.: Arch. Bioch., **25**, 191 (1950).
11. HAGEDORN, JENSEN: Biochem. Z., **137**, 92 (1923).
12. NEUHANS, O. W., LETZRING, M.: Anal. Chem., **29**, 1230 (1957).
13. BAUMANN—GRACE, J. B., KOVÁCS, A., TOMCSIK, J.: Schweiz. Z. allg. Path., **22**, 158 (1959).
14. IVÁNOVICS, G.: Z. Immun. Forsch., **97**, 402 (1940).

Address of the authors:

JUDIT LANTOS, GYÖRGY IVÁNOVICS

Institute of Microbiology, University Medical School, Beloiannis tér 10, Szeged, Hungary.