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Tomus XII

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ANTIGENIC STRUCTURE OF SAPROPHYTIC MYCOBACTERIA

By

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(Received June 17, 1964)

Summary. Saprophytic mycobacteria have been divided into three serological groups on the basis of immune-diffusion and immune-electrophoretic behaviour. *M. smegmatis*, *M. friburnsis* and *M. butyricum* fell into group I, *M. phlei* into group II and *M. pellegrino* into group III. Saprophytic mycobacteria differed in antigenic structure from *M. tuberculosis* and two atypical strains. Saprophytic strains can be identified and classified, and be distinguished from *M. tuberculosis* by immune diffusion and immune electrophoresis.

In view of the increasing number of "anonymous" acid-fast bacteria described in the literature, it seemed desirable to carry out further studies on the classification of these organisms. In addition to the classical morphological, biochemical and phage-typing methods, immune diffusion and immune electrophoresis may be of assistance in the classification of mycobacteria. It has been shown that when standard experimental conditions and immune sera are used, the results are comparable with those of other methods and yield an immunological basis for classification purposes [1—8].

These observations and the great number of unclassified acid-fast bacteria occurring in pure or mixed [9] culture in pathological materials, have made us to compare serologically some saprophytic mycobacteria, *M. tuberculosis* and two atypical strains presumably associated with human disease [10, 11].

Materials and methods

Mycobacterial strains. M. smegmatis (Budapest), *M. smegmatis* (Lausanne), *M. smegmatis* (U. S. A.), *M. butyricum* (Budapest), *M. friburgensis* (Budapest), *M. phlei* (Budapest), *M. phlei* (Lausanne), *M. pellegrino* (Budapest), H 37 Rv (Berlin), atypical strains 232 *(M. kansasii)* and 918 (Lausanne).

Preparation of immune sera. Ten-day Sauton cultures of *M. smegmatis* (Lausanne), *M. butyricum* (Budapest) *and M. phlei* (Lausanne) were treated with 1 per cent phenol for 12 hours at 37°C. The same treatment with 2 per cent phenol was applied for strains H 37 Rv and 232. The suspensions were then filtered, washed in sterile distilled water and dried at 4°C in the exsiccator. The dry bacteria were suspended in a sterile mixture containing 85 per cent Bayol 5 paraffin oil and 15 per cent Arlacel A, to give final concentrations of 100 mg/ml. Rabbits weighing 2200 to 2800 g were immunized subcutaneously with 1 ml weekly doses for 6 to 8 weeks. Before injecting the suspension, it was mixed with an equal volume of saline. The animals were exsanguinated 10 to 14 days after the last injection. The sera were passed through Seitz EK filters and stored at —10°C.

Preparation of antigens. Saprophytic strains were grown for 3 weeks, human and atypical strains for 6 weeks, in Sauton medium. After filtration in Büchner funnels, the culture was

centrifuged for 30 minutes at 6000 r.p.m. The supernatant was passed through G 5 glass filter, dialysed against distilled water at 4°C for 24 hours, then concentrated to 1/30 of the original volume under reduced pressure at 40°C. Standardization was performed by adding 0.9 per cent NaCl solution until the intensity of the biuret colour reaction corresponded to that given by a 30 mg/ml bovine albumin solution.

Immunological methods. (1) *Immune diffusion* as described by OUCHTERLONY [12]: 55°C agar prepared with pH 7.4 veronal sodium buffer was poured into Petri dishes, then wells were prepared and filled with undiluted immune serum and various dilutions of the antigen The plates were incubated in a wet chamber at 26°C for 5 days, then washed with saline, fixed with 2 per cent acetic acid, dried and stained with azocarmine B.

(2) *Immune electrophoresis* as described by GRABAR and BURTIN [13]: 55°C agar prepared with pH 8.2 veronal sodium buffer was poured into 12 × 12 cm glass plates, then wells were prepared and filled with the antigen mixed previously with buffered agar. Electrophoresis was carried out by use of interposed Seitz K5 and Whatman No. 1 papers with pH 8.2, ionic strength 0.1 veronal sodium buffer, at 3 V/cm potential drop for 4 hours. After electrophoresis other wells were prepared and filled with undiluted immune serum. The plates were then incubated in a wet chamber for 3 days at 26°C, washed with saline, fixed with a solution containing 50 per cent ethanol and 2 per cent acetic acid and finally stained with amidoschwarz 10 B and azocarmine B mixture (0.1 g amidoschwarz 10 B; 0.1 g azocarmine B; 45 ml 12 per cent acetic acid; 45 ml 1.6 per cent sodium acetate, 10 ml glycerol). Parallel with immune electrophoresis agar gel electrophoresis was carried out by the method of URIEL and GRABAR [14]. After the electrophoresis, proteins and lipids were stained with amidoschwarz 10 B and Sudan IV, respectively. SCHIFF's periodic acid reaction was also carried out.

(3) *Absorption experiments.* To 1 ml of immune serum 1 ml of heterologous antigen was added. The mixture was left to stand at 4°C overnight, then centrifuged for 30 minutes at 7000 r.p.m. The supernatant was used as an absorbed serum.

Results

Examination of immune sera in homologous immune precipitation systems. Concentrated filtrates of mycobacterial cultures were examined with the homologous sera. With anti-smegmatis serum 13 antigen components were revealed in each *M. smegmatis* strain. The majority of components migrated towards the anode in characteristic groups, sometimes in an almost perfectly

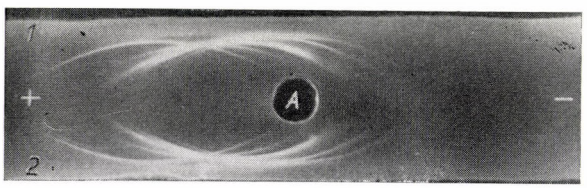

Fig. 1. Immune electrophoresis of *M. smegmatis* (Lausanne) antigen (well A) *vs.* anti-smegmatis serum (wells 1,2)

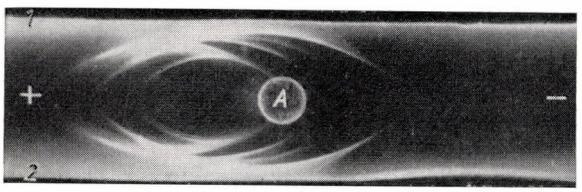

Fig. 2. Immune electrophoresis of *M. phlei* (Lausanne) antigen (well A) *vs.* anti-phlei serum (wells 1,2)

unchanged position. Two components migrated towards the cathode (Fig. 1). With the homologous immune sera in *M. phlei* 11 (Fig. 2), in *M. butyricum* 10 antigen components were shown. With the exception of one component in

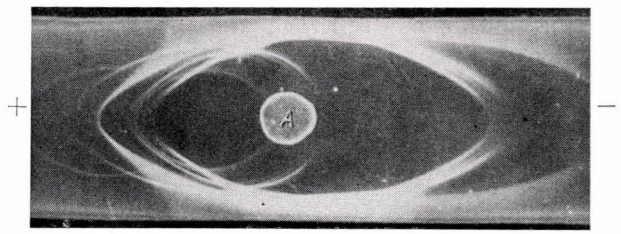

Fig. 3. Immune electrophoresis of atypical strain 232 antigen (well A) *vs.* anti-232 serum (wells 1,2)

Fig. 4. Agar gel electrophoresis of *M. smegmatis* antigen. Schiff's periodic acid staining

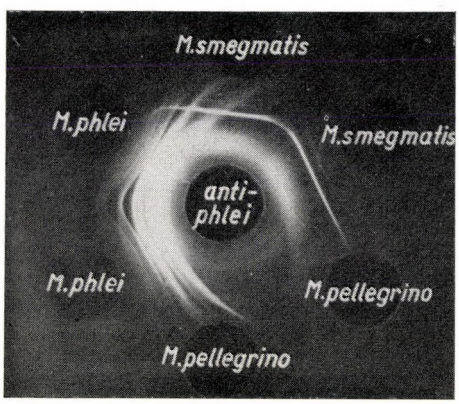

Fig. 5. Immune-diffusion pattern of *M. phlei*, *M. smegmatis* and *M. pellegrino* antigens *vs.* anti-phlei serum

each of the latter strains, these components migrated towards the anode. In each of strains H 37 Rv and 232, 10 components were found; these strains contained more positively charged components than saprohytic mycobacteria (Fig. 3). The cultures showed an optimum protein staining corresponding to the antigenic groups. With Sudan IV, strains H 37 Rv and the atypical strains

showed an optimum staining in the wells. In antigenic extracts prepared from saprophytic mycobacteria no such staining was observed. With SCHIFF's reaction human and atypical strains were characterized by one optimum in the well and another in the direction of the cathode. In saprohytic organisms

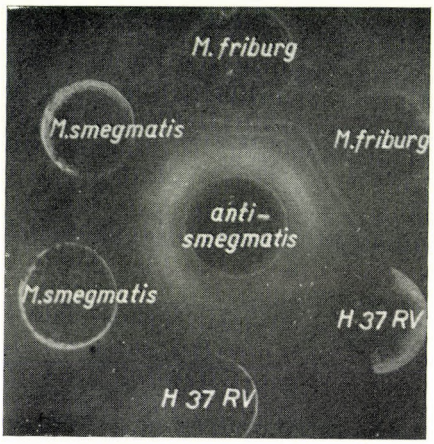

Fig. 6. Immune-diffusion pattern of *M. smegmatis*, *M. friburgensis* and H 37 Rv antigens *vs.* anti-smegmatis serum

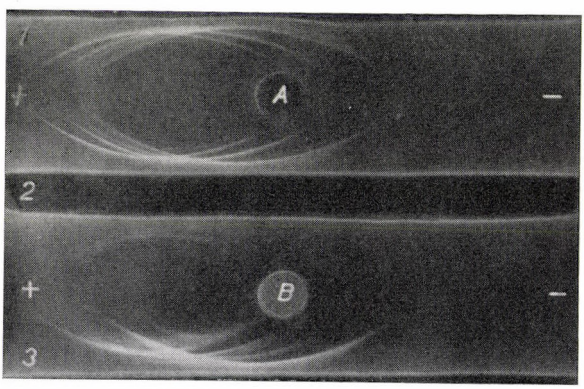

Fig. 7. Immune electrophoresis of *M. butyricum* (well A) and *M. phlei* (well B) antigens *vs.* antibutyricum (wells 1,2) and anti-phlei (well 3) sera

only one SCHIFF-positive optimum migrating towards the cathode was revealed. The only exception was *M. phlei*, which contained no SCHIFF-positive component (Fig. 4).

Examination of immune sera in heterologous immune precipitation systems. Comparison of the antigenic structure of saprophytic mycobacteria and demonstration of common antigens were carried out in several experiments. For immune diffusion and immune electrophoresis, antigens prepared at

different times were employed. In order to assure an optimal reaction for every antigen component, the antigens and sera were tested at various concentrations. It was shown that *M. smegmatis* strains were antigenically identical. This species shared 11 and 10 common antigen components with *M. friburgensis* and *M. butyricum*, respectively. The common components were identical as to serological behaviour and electrophoretic mobility. As between *M. smegmatis* and *M. friburgensis* or *M. butyricum* a slight serological difference was noted, serum anti-smegmatis was absorbed with *M. friburgensis* and *M. butyricum*.

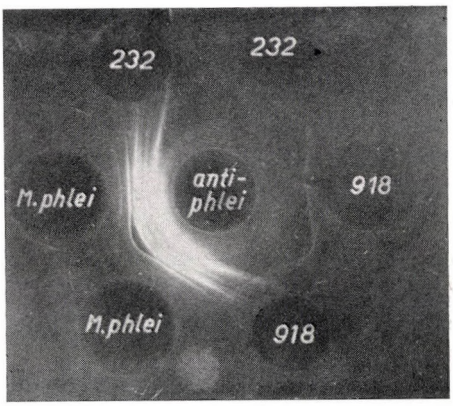

Fig. 8. Immune-diffusion pattern of antigens prepared from *M. phlei* and atypical strains, *vs.* anti-phlei serum

Since these experiments did not confirm the supposed difference, these species were included in one immunological group. In antigenic structure our 2 *M. phlei* strains were identical, but differed considerably from the former cultures. *M. phlei* was therefore regarded as comprising a separate serological group. Our *M. pellegrino* strain shared only one antigen component with other mycobacteria. This factor corresponded to the component common to strain H 37 Rv and saprophytic cultures (Figs. 5, 6, 7).

Antigenic difference among saprophytic, M. tuberculosis and atypical strains. Examination in heterologous immune precipitation systems showed that *M. tuberculosis* and the atypical strains differed principally in antigenic structure from saprohytic cultures. Some common antigens, however, were demonstrated (Fig. 8.).

Discussion

Large doses of saprophytic mycobacteria given with incomplete Freund adjuvant produce broad precipitin spectrum immune sera. In the present experiments dialysed and concentrated filtrates of mycobacterial cultures were applied as antigen extracts. In the preparations at least 10 different

components were present. On the basis of serological identity and electrophoretic mobility of their component, ssaprophytic mycobacteria were divided into 3 serological groups: I, *M. smegmatis*, *M. friburgensis* and *M. butyricum*; II, *M. phlei*; III, *M. pellegrino*. Common antigen components revealed in the 3 groups were identical as to serological behaviour and electrophoretic mobility. *M. tuberculosis* and the 2 atypical strains differed considerably from saprophytic mycobacteria, although some common components were present in all of these organisms. Absorption experiments revealed no difference among various strains and substrains of species belonging to group I. The 2 *M. phlei* strains falling into group II were identical in antigenic structure. From the results it may be concluded that saprophytic mycobacteria can be identified and classified by immune-diffusion and immune-electrophoresis and be differentiated from *M. tuberculosis* and atypical strains.

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SALICIN-FERMENTING VARIANT OF SHIGELLA FLEXNERI SEROTYPE 2a

By

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(Received September 18, 1964)

Summary. Parallel subcultures of an organism antigenically identical with *Sh. flexneri* 2a yielded sorbitol-positive *Sh. flexneri* 2a variants, rhamnose positive cultures and salicin-fermenting Shigella-coli intermediary strains. The strain isolated from a patient with clinical symptoms of dysentery, caused keratoconjunctivitis shigellosa in the guinea pig.

In the Dysentery Laboratory of our institute some quick-sorbitol-fermenting *Shigella flexneri* 2a cultures were isolated from routine material. The first, non-selected subcultures of these strains fermented rhamnose rapidly or slowly in 0.5—100 per cent. Some subcultures of strains 215 and 295, which had been isolated on different occasions from the same patient also fermented salicin. As according to the decision of the International Enterobacteriaceae Subcommittee, salicin-fermenting organisms should be excluded from the Shigella group, and to our knowledge no such *Sh. flexneri* strains have been described, it seemed desirable to perform detailed studies on the taxonomic position of our cultures.

Materials and methods

Generally the methods recommended by the *International Enterobacteriaceae Subcommittee* [1], EDWARDS and EWING [2], KAUFFMANN [3] and RAUSS [4] were used. Most of these methods are routinely employed in our laboratory [5—7].

Colonial morphology was examined in the usual manner at reflected light and also at transmitted oblique artificial illumination [8].

Biochemical reactions. Fermentation of salicin was tested by use of various commercial and one recrystallized preparation. Purity of the latter substance was checked by melting point determination. Salicin was dissolved at 0.5 per cent concentration in peptone water containing bromcresol purple indicator or in KAUFFMANN's peptone water. Fermentation was examined at least in 100 parallel tubes at 37°C. Observation lasted for 30 days or until acid reaction had appeared. In addition to lactose, beta-galactosidase activity was tested with *o*-nitrophenyl-beta-D-galactoside (ONPG) [9—12].

Serological examinations. E. coli O13 and Alkalescens-Dispar strains were supplied by the Culture Collection Centre of our institute, which obtained the former strain from the State Serum Institute, Copenhagen, and the latter cultures from the Public Health Laboratory Service, London. Rabbit immune sera were prepared in our laboratory.

Testing of virulence was performed by conjunctival infection of guinea pigs [13]. Purity of the cultures was ensured by several passages on culture media, or, in case of virulent strain 215, also by serial inoculation into the eyes of guinea pigs.

Results

In colonial morphology the two strains resembled shigellae. When subcultured on yeast extract agar after a longer storage on Dorset medium, the cultures produced optically inhomogeneous, "reticular" colonial variants (14).

Table I

Biochemical properties of strains 215 and 295

Arabinose	+[1]	Ammonium glucose	—
Xylose	—	Ammonium citrate	—
Rhamnose	×[1–28]	Malonate	—
Glucose	+[1]	Mucate	—
Sucrose	—		
Lactose	—	Lysine	—
Maltose	+[1]	Arginine	—
		Ornithine	—
Mannitol	+[1]	Glutamic acid	+
Sorbitol	+[1]	Carlquist's reaction	—
Adonitol	—		
Dulcitol	—		
Inositol	—		
Salicin	×[1–28]	Tryptophan deaminase	—
Beta-galactosidase (ONPG)	—	Phenylalanine deaminase	—
Indole	+		
Methyl red	+	Motility	—
Voges-Proskauer	—		
H_2S	—	Keratoconjunctivitis reaction	
Urease	—	in guinea pigs: 215	+
Gelatin	—	295	—
KCN	—		

In checking the purity of subcultures the examination of these variants was also included. The cultures consisted of Gram negative rods showing no motility in semisolid (0.2 per cent) agar.

Strain 215, which was isolated first, proved highly virulent in the guinea pig eye. Strain 295 isolated 9 weeks later, was non-virulent.

The two strains were similar in antibiotic sensitivity. They were resistant to penicillin, streptomycin, erythromycin and sulphonamides, and sensitive to tetracyclines, chloramphenicol, neomycin and polymyxin.

The biochemical behaviour of the strains is summarized in Table I. Salicin and rhamnose fermentation results were obtained with the first parallel subcultures of strains isolated directly from the faecal specimens. Subcultures

Table IIa

Serological analysis of cultures 215, 295, Sh. flexneri 2a and Alkalescens-Dispar

Serum	Absorbed by	Strain						
		215	295	2a	A—D 01	A—D 02	A—D 03	A—D 04
215	—	3200	3200	1 600	10	20	100	400
	215	—	—	—	—	—	—	—
	295	—	—	—	—	—	—	—
	2a	—	—	—	—	—	—	—
	A—D 01	1600	1600	1 600	—	200	—	400
	02	1600	1600	1 600	—	—	—	400
	03	1600	1600	1 600	—	20	—	400
	04	800	800	800	—	20	—	—
295	—	1600	1600	6 400	200	200	200	100
	215	—	—	—	—	—	—	—
	295	—	—	—	—	—	—	—
	2a	—	—	—	—	—	—	—
	A—D 01	800	800	800	—	100	—	100
	02	800	800	400	400	—	—	100
	03	800	1600	800	400	20	—	20
	04	800	1600	800	400	20	—	—
2a	—	1600	1600	12 800	10	200	10	200
	215	—	—	—	—	—	—	—
	295	—	—	—	—	—	—	—
	2a	—	—	—	—	20	—	20
	A—D 01	3200	1600	3 200	—	200	—	100
	02	800	1600	1 600	—	—	—	100
	03	800	1600	800	—	—	—	100
	04	1600	1600	1 600	—	—	—	—

of strain 215 fermented salicin and rhamnose in about 10 per cent; those of strain 295 fermented rhamnose in 10, while salicin only in 0.5 per cent. All subcultures of both strains split sorbitol. By adaptation and selection of positive subcultures, variants producing acid after 1 day in rhamnose and/or salicin, were obtained. As to other biochemical properties and antigenic struc-

ture, the rhamnose and salicin-positive variants resembled the original cultures or their salicin-negative variants. Neither of the variants split lactose in 30 days or ONPG in 20 hours. Variants cultured on agar containing 10 per cent lactose [12] displayed no beta-galactodidase activity. As regards fermentation reactions, our strains reminded mostly of lactose-negative coli strains or

Table IIb

Serological analysis of cultures 215, 295, Sh. flexneri 2a and Alkalescens-Dispar

Serum	Absorbed by	Strain			Serum	Absorbed by	Strain		
		215	295	2a			215	295	2a
	—	20	20	10		—	—	10	10
	215	—	—	—		215	—	—	—
A—D 01	295	—	—	—	A—D 03	295	—	—	—
	2a	—	—	—		2a	—	—	—
	A—D 01	—	—	—		A—D 03	—	—	—
	—	20	20	20		—	40	40	20
	215	—	—	—		215	—	—	—
A—D 02	295	—	—	—	A—D 04	295	—	—	—
	2a	—	—	—		2a	—	—	—
	A—D 02	—	—	—		A—D 04	—	—	—

some salicin-positive members of the Alkalescens-Dispar group. Their other biochemical properties were characteristic of shigellae.

As determined by slide agglutination with factor sera prepared according to RAUSS and according to EWING, both strains gave reactions typical of *Sh. flexneri* 2a; D++++, $VIII_2$++++, $VIII_{1.2\,3}$++ (RAUSS), or II++++, 3, 4++++ (EWING).

In view of slide agglutination reactions, our strains were to be distinguished primarily from *E. coli* 013 and Alkalescens-Dispar serotypes. With *E. coli* 013 our strains gave a definite (1600—6400) partial reaction corresponding to that obtained with type strain *Sh. flexneri* 2a. The antigenic relationship between *E. coli* 013 and *Sh. flexneri* 2a is known from the literature.

Of Alkalescens-Dispar strains those belonging to FRANTZEN's 01, 02, 03 and 04 groups gave cross agglutination with our strains.

Results of the serological analysis are summarized in Tables II a, b, and c. The antigenic identity of the examined strains and *Sh. flexneri* 2a is evident:

(1) In cross absorption experiments sera for our cultures and *Sh. flexneri* 2a were completely depleted of agglutinins against any of the absorbing strains and also against Alkalescens-Dispar serotypes.

(2) Absorption of the above sera by Alkalescens-Dispar strains caused no significant decrease in titres. Thus the type-specific agglutinin content was not affected.

(3) Alkalescens-Dispar sera contained low titre agglutinins against our strains and *Sh. flexneri* 2a; these were completely removed after absorption by the same cultures.

(4) Finally, the specific agglutinability of Alkalescens-Dispar strains in the homologous antisera remained unaltered after absorption by strains 215,

Table IIc

Serological analysis of cultures 215, 296, Sh. flexneri 2a and Alkalescens-Dispar

Serum	Absorbed by	Strain				Serum	Absorbed by	Strain			
		A–D 01	A–D 02	A–D 03	A–D 04			A–D 01	A–D 02	A–D 03	A–D 04
A–D 01	—	6400	40	—	20	A–D 03	—	—	400	3200	200
	215	6400	—	—	—		215	—	800	—	200
	295	6400	—	—	—		295	—	400	—	200
	2a	3200	—	—	—		2a	—	800	—	200
	A–D 01	—	—	—	—		A–D 03	—	—	—	—
A–D 02	—	—	12 800	—	200	A–D 04	—	20	400	—	6400
	215	—	12 800	—	400		215	—	800	—	3200
	295	—	6 400	—	400		295	—	400	—	3200
	2a	—	6 400	—	400		2a	—	800	—	6400
	A–D 02	—	—	—	—		A–D 04	—	—	—	—

295 or *Sh. flexneri* 2a. The only exception — Alkalescens-Dispar strain 03 in the homologous serum — may be explained by an alteration in the antigens of the strain, which is an old laboratory culture. This finding bears no taxonomical importance, as in this respect our strains behaved identically with type strain *Sh. flexneri* 2a.

Discussion

Strains 215 and 295 are identical in antigenic structure with *Sh. flexneri* 2a and show all the known serological relationships existing among this serotype and other bacteria [2—4]. Despite their complete serological identity, it is questionable whether our strains correspond to *Sh. flexneri* 2a or to a new *E. coli* 0 group.

Between the Shigella and Escherichia groups several serological cross reactions have been noted. Some coli cultures contain complete Shigella anti-

gens, like the strain representing *E. coli* O group 129 [15], which is antigenically identical with *Sh. flexneri* 5. The strain isolated by RAUSS and VERTÉNYI [16] and later described as type strain *E. coli* O group 135, is serologically identical with *Sh. flexneri* 4b. BRANDES [17] and SLOPEK and DABROVSKY [18] reported on coli strains sharing common antigens with *Sh. flexneri* 5 or 3.

Strains of doubtful taxonomic position have also been described. These cultures, isolated from patients with dysentery or healthy individuals, remind mostly of hybrids obtained in recombination experiments. Such cultures causing keratoconjunctivitis in guinea pigs were isolated by MANOLOV [19]. These strains were reinvestigated by STENZEL [20], but the problem of their classification has not yet been solved. Similar strains were examined by SZTURM—RUBINSTEN (Saigon strains) which shared antigens with *Sh. boydii* 11 and Alkalescens-Dispar O group 4 [21].

With the exception of some fermentation reactions mentioned above, strains 215 and 295 were biochemically typical Shigella cultures. On the basis of their sorbitol and rhamnose positivity our strains cannot be excluded from *Sh. flexneri* 2a, as such organisms, although infrequently, occur within this serotype [2]. The only property which suggests that the cultures may not belong to the Shigella group, is its salicin positivity. The salicin positive-cultures have, however, been shown to split off large numbers (80—90 per cent) of variants which are indistinguishable from the known sorbitol-positive biotype of *Sh. flexneri* 2a. It should be noted that SERÉNY [22], SIROKO [23], NAKAMURA [24], KLECKOVA-ALDOVA [25] showed that a significant part of otherwise typical *Sh. sonnei* strains produced salicin-positive variants. Salicin-fermenting variants of our strain 215 isolated first, similarly to salicin-negative cultures, gave rise to typical keratoconjunctivitis shigellosa. Therefore, in view of their serological, biochemical and pathogenic properties, our strains should be regarded as salicin-fermenting variants of *Sh. flexneri* 2a. These strains are able to produce intermediate cultures as sorbitol-positive biovariants of *Sh. flexneri* 2a, rhamnose-positive organisms and salicin-positive Shigella-coli intermediary forms.

Intermediary forms may be produced by spontaneous mutation. This holds especially true for very rarely encountered cultures. On the other hand, it has been demonstrated *in vitro*, that unusual variants can be produced by phage action or by direct crossing. The lactose-positive *Sh. flexneri* obtained in transduction experiments and our salicin-positive cultures are similar in character. Both kinds of positive variants are partly unstable, split off negative cultures easily and some of their surface cultures, when stored in the refrigerator, lose their atypical fermentation capacity [26]. It is remarkable that the antigenic structure of the original *Sh. flexneri* culture should be preserved mainly in the stable lactose-fermenting subcultures of unstable hybrids [27].

These considerations only serve as an aid for further orientation and, of course, cannot be regarded as the solution of the problem.

Acknowledgement. The author is indebted to Dr. B. LÁNYI, Head of Culture Collection Centre, State Institute of Hygiene, for supplying the type strains.

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OBSERVATIONS ON BIOCHEMICAL CHANGES IN IRRADIATED SPORES OF BACILLUS CEREUS

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Summary. Dipicolinic acid content of resting *Bacillus cereus* spores contained in a dense aqueous suspension has been investigated after irradiation. Spectrophotometry and chromatography were applied to the study of substances leaking from bacterial cells after irradiation with lethal doses.

It was established that the "pseudogermination" observable in the resting spores after irradiation with high doses is correlated to the increased permeability of the cell wall. It appears that much higher doses are required to damage the cytoplasm membrane than to desorganize the processes inducing cell division.

The substances leaking from the spore as a result of irradiation resemble those characterizing the exudate of physiological germination.

The high resistance of bacterial spores to bactericide factors constitutes a serious problem in many branches of industry and is of utmost importance in the practical realization of radiation sterilization for food preservation.

The knowledge of the fundamental biochemical factors determining the state and radiation resistance of spores may help to influence these factors advantageously, to lower the radiation resistance of the spores.

In a previous paper [3] it was stated that on irradiation with X-rays at a high dosage level, in a resting spore suspension of *Bacillus cereus* a significant increase of germinated *(i.e.* dark) spores could be observed. Since the phenomenon termed "pseudo-germination" is probably due to an increased permeability of the cell wall, experiments have been carried out in order to study the biochemical changes induced by irradiation in *B. cereus* suspensions.

Materials and methods

The *B. cereus* strain used was the same as in the previous experiments. The methods applied for propagation and in the production of vegetative cell masses, germinating and resting spores, respectively, have been described previously [3].

Methods of irradiation. Irradiation was effected with a type "Stabil 250" X-ray apparatus, operated at 250 kVp and 15 mA, without filter.

The cells to be irradiated were suspended in sterile distilled water. The density of the cell suspension was set at the desired level by microscopic cell count determinations. Irradiation was carried out at room temperature but when long periods of irradiation were applied a warming up of the samples to 30° C was to be expected.

Dipicolinic acid (DPA) content in irradiated or heat-treated samples was determined by the method of JANSSEN *et al.* [5] using a Unicam SP 500 type spectrophotometer.

Absorption spectra in the UV range of the clear supernatants of the centrifuged suspensions of bacteria were determined with the aid of the Unicam SP 500 spectrophotometer.

Ninhydrin-positive compounds were determined by mixing 1 ml samples of the supernatants with 4.3 ml of a ninhydrin-containing citrate buffer of pH 5.0 and placing the mixture for 10 minutes into a boiling water bath [17]. After cooling the sample, its colour intensity was determined in a Pulfrich stepphotometer using filter S 574.

Paper and thin-layer chromatography of the samples was carried out as follows.

Five ml samples of the supernatants of bacterial suspensions of 10^{10} ml density were evaporated in a water bath, the remainder was taken up in 0.1 ml distilled water (thus forming a fiftyfold concentrate).

For amino acid paper chromatography about 20 μl of the concentrate was dropped on Whatman's No. 1 paper and subjected to descending chromatography for 20 hours, at room temperature. The solvent system consisted of n-butyl alcohol-acetic acid-water (120 : 30 : 50, v/v) [15].

The chromatograms were developed by spraying with the ninhydrin containing solution as suggested by MOFFAT and LYTLE [6], and dried at 100°C during 10 minutes.

In two-dimensional paper chromatography of amino acids the solvent system used in the first dimension was n-butyl alcohol — acetic acid — water, and in the second dimension a phenol — ammoniumhydroxide mixture according to SMITH [15].

Thin-layer chromatography was carried out in a silica-gel adsorbent (Kieselgel "G", Merck), without activation [2]. For the separation of amino acids several solvent-systems were tested; of these the following proved to be the best.

A. n-butyl alcohol — acetic acid — water (80 : 20 : 20, v/v)

B. phenol-water, (75 : 25, w/w)

C. n-propyl alcohol — 25 per cent ammonium hydroxide, (70 : 30, v/v).

When the solvent-front reached a distance of 10 cm from the start-line the run was interrupted.

In the first dimension of the two-dimensional thin-layer chromatography solvent A, in the second, solvent B was used. Thin-layer chromatograms were developed in the same way as the paper chromatograms.

Results

Dipicolinic acid content of irradiated or heat-treated spores. The suspensions of resting spores (at a bacterial density of approximately 10^{10}/ml) were

Table I

Dipicolinic acid content of irradiated or heat-treated resting spores

X-ray dose or heat-treatment	Concentration of dipicolinic acid DPA μg/10^9 spores	Relative concentration of DPA %
0 krad (untreated)	34.8	100.0
250 krad	35.9	103.4
500 krad	33.9	97.2
1000 krad	30.8	88.3
2000 krad	11.9	34.1
100°C, 30 minutes	3.2	9.3

irradiated at different dose levels or heat-treated at 100°C for 30 minutes and after centrifugation and washing with distilled water, the dipicolinic acid content was determined. Results of these investigations are given in Table I.

The dipicolinic acid content of germinated spores produced as described previously [3] was about 42 per cent of that of resting spores.

The absorption spectra and DPA-content of the supernatants of irradiated bacterium suspensions. Many investigations have proved the disorganizing effect of various chemicals and heat-treatment on the cytoplasmic membrane [4, 12, 13, 16]. VAS and FARKAS [18] observed such a direct effect of ionizing radiation on yeast cells. The spore germination stimulating effect of high radiation doses is presumably due to the increased permeability of the cell wall, causing the leakage of various cell components into the surrounding medium.

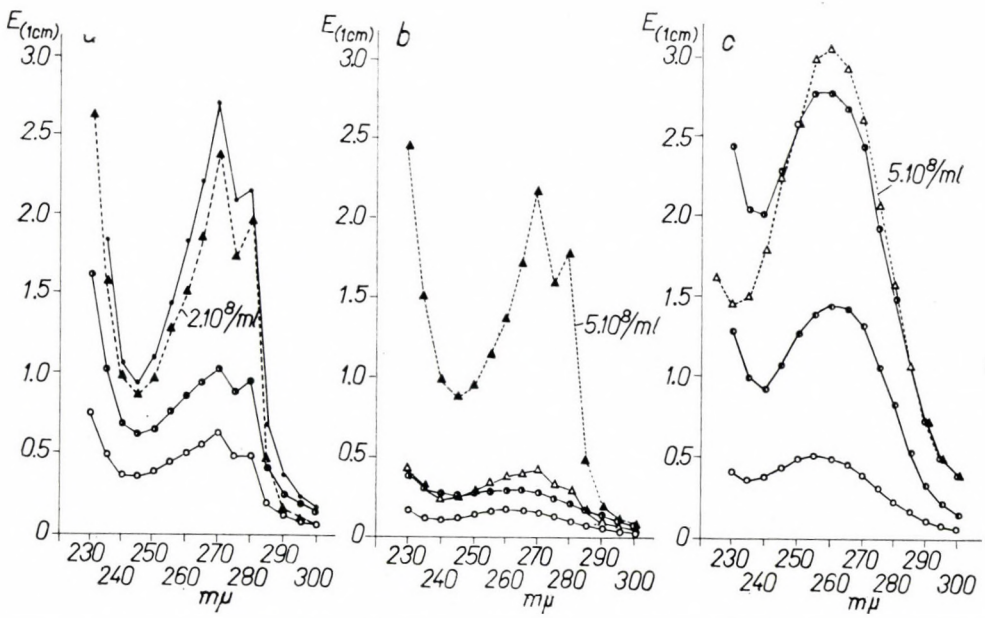

Fig. 1. Absorption spectra in the ultraviolet range of supernatants of *Bacillus cereus* suspensions after irradiation and heat-treatment, respectively, *a)* diagram: resting spores, *b)* diagram: germinated spores, *c)* diagram: vegetative cells

○ 0 krad; ◐ 500 krad; ◑ 1000 krad; ● 2000 krad; △80°C, 10 minutes; ▲ 100°C, 30 minutes

In order to establish indirectly the changes in permeability, dense suspensions (about 10^{10} cells/ml) of resting and germinated spores and vegetative cells, respectively, were centrifuged after irradiation or heat-treatment, and the clear supernatants were investigated spectrophotometrically in the ultraviolet region. Some of the spectra measured against distilled water are shown in Fig. 1.

In Fig. 1 the continous curves indicate irradiated suspensions with a microbial density of 10^9/ml. The dotted lines correspond to the spectra of supernatants of heat-treated suspensions at the indicated cell densities. As it may be seen, the supernatants of resting cell suspensions have two peaks at 270

and 280 mμ, respectively, whereas that of the vegetative cell suspension has a single peak at 260 mμ. The spectra of the supernatants of germinated cell

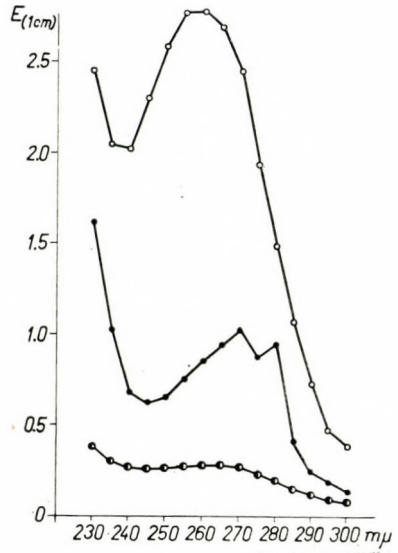

Fig. 2. Absorption spectra in the ultraviolet range of resting spores, germinated spores and vegetative cells

● resting spores, irradiated at 1000 krad; ◐ germinated spores, irradiated at 1000 krad; ○ vegetative cells, irradiated at 1000 krad

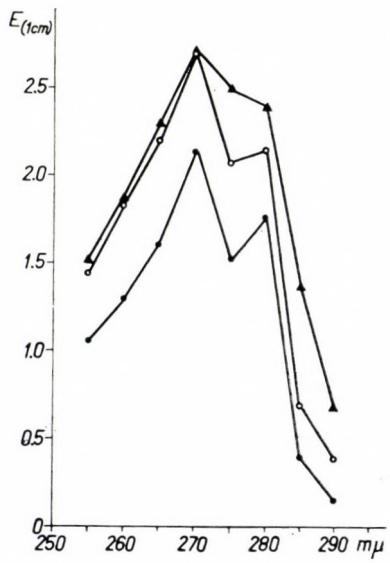

Fig. 3. ▲ Dipicolinic acid solution of 100 μg/ml concentration

○ The supernatant of a resting spore suspension of 10^9/ml cell density, irradiated at 2000 krad; ● An aqueous solution containig 100 μg/ml DPA and 560 μg/ml $Ca(NO_3)_2 4H_2O$. (The absorption in the UV range of the $Ca(NO_3)_2$ solution of given concentration is negligible)

suspensions show after irradiation the character of vegetative cells, and after heat-treatment that of resting cells.

The typical curves, each belonging to one of the suspensions of resting, germinating and vegetative cells, may be compared directly in Fig. 2.

The absorption spectrum with two peaks, characteristic of resting spores, corresponds to the spectrum of Ca-dipicolinate, the representative component of bacterial spores (Fig. 3).

Since the height of the curves could be influenced by other substances present in the supernatants it was attempted to characterize the amount of

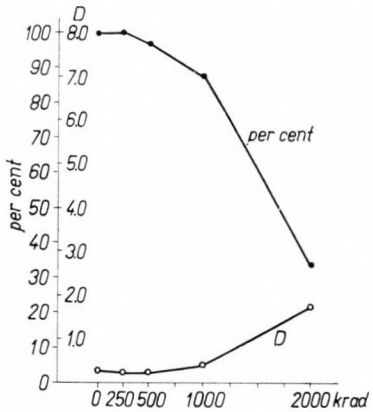

Fig. 4. DPA concentration in the cells and in the medium, under the effect of irradiation and heat-treatment, respectively
per cent = relative DPA concentration in the resting spores according to Table I.
D = difference between the values obtained at 270 mμ and 245 mμ, respectively, in cell suspensions of 10^9/ml density

dipicolinate leaking from the cells by the difference (D) between the maximum value measured at 270 mμ and the minimum value measured at 245 mμ.

D values as a function of the radiation dose are shown in Fig. 4. The curve belonging to the relative amount of dipicolinic acid remaining in the spores, based on data given in Table I, is also presented in Fig. 4.

As seen in Fig. 4, the leakage of dipicolinate into the environment strongly increases after irradiation at the 500—1000 krad level. The results of both methods seem to prove that, during heat-treatment at 100°C for 30 minutes, about 90 per cent of the dipicolinic acid will leake into the environment. However, the dipicolinate content of the environment does not increase to the same extent as the content of the irradiated spores decreases. It seemed probable that part of the dipicolinic acid suffered degradation as a result of irradiation. Therefore the behaviour of pure dipicolinic acid, *i.e.* Ca-dipicolinate during irradiation was also investigated (Fig. 5). It was established that the aqueous

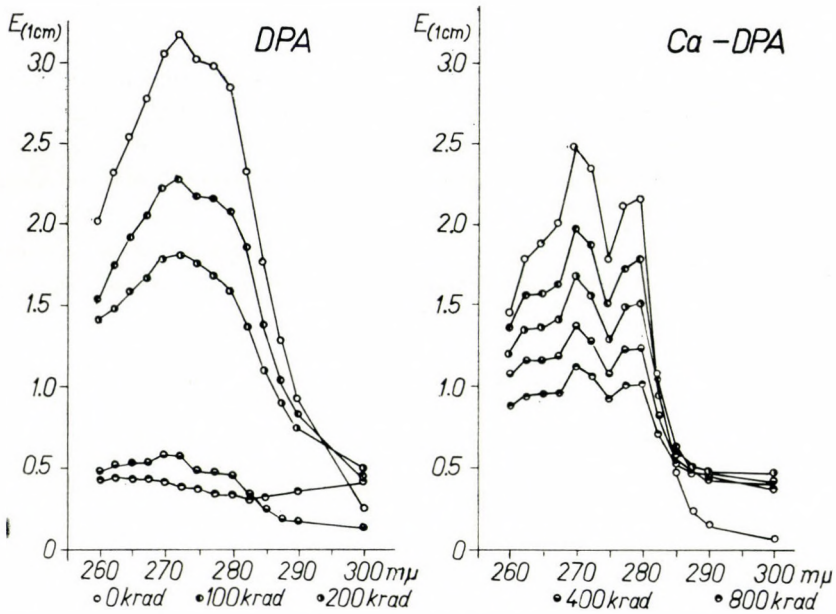

Fig. 5. Ultraviolet spectrum of solutions containing 100 μg/ml DPA and 100 μg/ml DPA + + 560 μg/ml $Ca(NO_3)_2 4H_2O$, as affected by irradiation

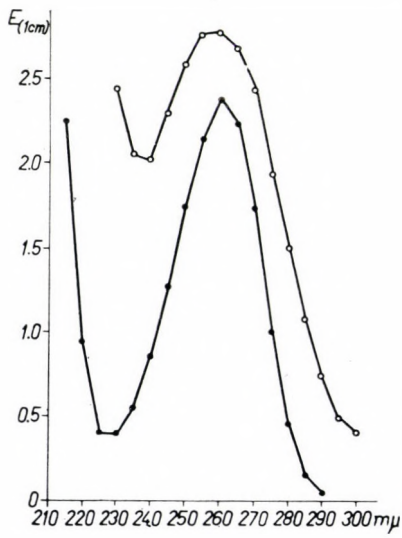

Fig. 6 Absorption spectrum of supernatant of a vegetative cell suspension and of aqueous adenosine solution

○ Supernatant of a vegetative suspension of 10^9/ml cell density, irradiated at 1000 krad.
● Aqueous solution containing 45 μg/ml adenosine

solution of dipicolinic acid was rather sensitive to radiation. The characteristic maximum of dipicolinic acid disappears when irradiated above the 400 krad level. The chelate formed with Ca seems to be more resistant in the aqueous solution. Although optical density values decreased with the increase of the

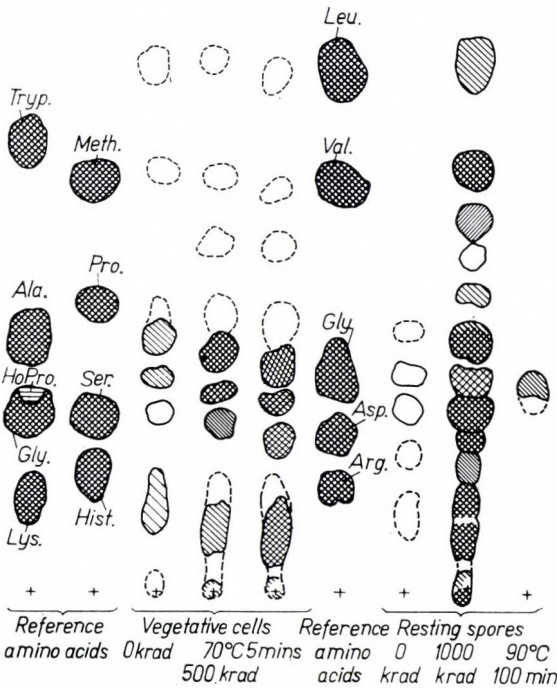

Fig. 7. The copy of a one-dimensional paper-chromatogram

Legend to abbreviations on the chromatograms: Ala. = alanine; β-Ala. = β-alanine; Arg. = arginine; Asp. = aspartic acid; BuA = n-butyl alcohol—acetic acid — water; Cys. = cystine; CysA. = cysteic acid; Cyste. = cysteine; Glu. = glutamic acid; Gly. = glycine; Hist. = histidine; Ho-Pro. = hydroxy-proline; Leu. = leucine; i-Leu. = iso-leucine; Lys.= lysine; Me.s. = methionine sulphonate; Meth. = methionine; Ph.-NH_3 = phenol-ammonium-hydroxide; Pro. = proline; Ser. = serine; Threo. = threonine; Tryp. = tryptophan; Tyro = tyrosine

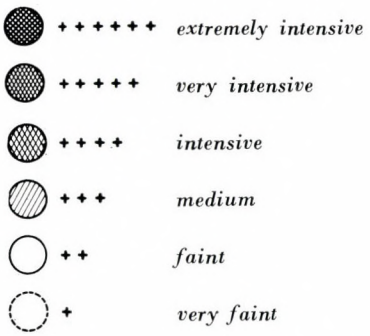

Fig. 8. Intensity of ninhydrin-positive spots in schematic drawing and tables based on the chromatogram

irradiation levels, the character of the absorption curves after irradiation at or above 800 krad did not differ from that of the untreated samples. From the results obtained it seems probable that dipicolinic acid is protected, in addition to calcium, by other cell components as well. Thus the total dipicolinic acid content of the cells and the medium begins to decrease only after treatment

Fig. 9. Thin-layer chromatogram of supernatant of vegetative and resting spore suspensions. 1. Vegetative cells, 0 krad; 2. Vegetative cells, 100 krad; 3. Vegetative cells, 500 krad; 4. Vegetative cells, 70°C, 5 minutes; 5. Resting spores, 0 krad; 6. Resting spores, 500 krad; 7. Resting spores, 1000 krad; 8. Resting spores, 90°C, 100 minutes; 9., 10., 11. Amino acid solutions for comparison

with 1000 krad and even in samples irradiated with 2 Mrad nearly two-third of the original DPA content is to be found.

The absorption spectra in the UV region of pure solutions showed a good agreement with the results obtained by other authors [9, 14]. The results of the present experiments corresponded well to the observations of SLEPECKY in that the optical density values belonging to the calcium chelate are much lower than those belonging to a dipicolinic acid solution of equal concentration.

The principal components determining the character of the supernatants of vegetative cell suspensions are probably the purine and pyrimidine derivatives. This is shown by Fig. 6 in which the absorption spectra of an adenosine solution and that of the supernatant of an irradiated vegetative cell suspension are compared. As an effect of surface active compounds [12, 13] and other chemicals [1], purines and pyrimidines will leak from Gram-positive microorganisms.

Table II

Paper chromatographic analysis

One-dimensional, descending chromatography; solvent system: n-butyl alcohol—acetic acid—water (120 : 30 : 50 v/v)

Components	RF values (%)		Supernatants					
	literary (15)	authors	Vegetative cells			Resting spores		
			0	500 krad	70° C 5 minutes	0	1000 krad	90° C 100 minutes
	results							
cystine	5						+++	
cysteine	8					+	++++	
histidine	11	14						
lysine	12	11	++	+++	++++	+	++++	
arginine	15	13						
hydroxy-proline	22	23						
aspartic acid	23	20						
serine	22	21	++	+++	++++	++	++++++	+
glycine	23	21						
threonine	26							
glutamic acid	28	26	+++	++++++	++++++	++	+++++	+++
alanine	30	30	+++	+++++	+++++	+	+++++	
β-alanine	33		+	+	+		++	
proline	34	35						
γ-amino-n-butyric acid	40			+	+		++	
tyrosine	45						+++	
methionine	50	50						
tryptophan	50	54	+	+	+	+	++++	
valine	51	50						
iso-leucine	67							
leucine	70	63	+	+	+		++++	

Table III

Thin-layer chromatographic analysis

One-dimensional, ascending chromatography; solvent system: phenol—water, (75 : 25 w/w)

Components	Rf values (%)		Supernatants							
	literary (2)	authors	Vegetative cells				Resting spores			
			0	100	500	70° C 5 minutes	0	500	1000	90° C 100 minutes
	results		krad				krad			
cysteic acid	4		+	+	+++	++	++	+++	+++	+
aspartic acid	6	12								
lysine	9	9			++++	++++			+++++	
glutamic acid	10	12								
arginine	19	21								
serine	20	15	+	+	++++	++	++	++	++++	++
glycine	24									
threonine	26									
alanine	29	25			+++++	++++		+++	++++++	
β-alanine	30									
histidine	32	23			++	+		++	+++	+
hydroxy-proline	38	32								
valine	40	32								
nor-valine	42									
tyrosine	47									
leucine	48	42	+	+	++	+	+	++	+++++	+
iso-leucine	49									
methionine	49	39								
proline	50	41								
tryptophan	63	53								

Ninhydrin-positive compounds in the supernatants. Irradiation effected also the leakage from the cells of ninhydrin-positive compounds and these were investigated by chromatography. The results of the investigations are shown in Fig. 7 and Table II. The intensity of spots of ninhydrin-positive substances is indicated in Fig. 8.

For the analysis of ninhydrin-positive compounds present in the supernatants, thin-layer chromatography also proved to be suitable. Fig. 9 shows a

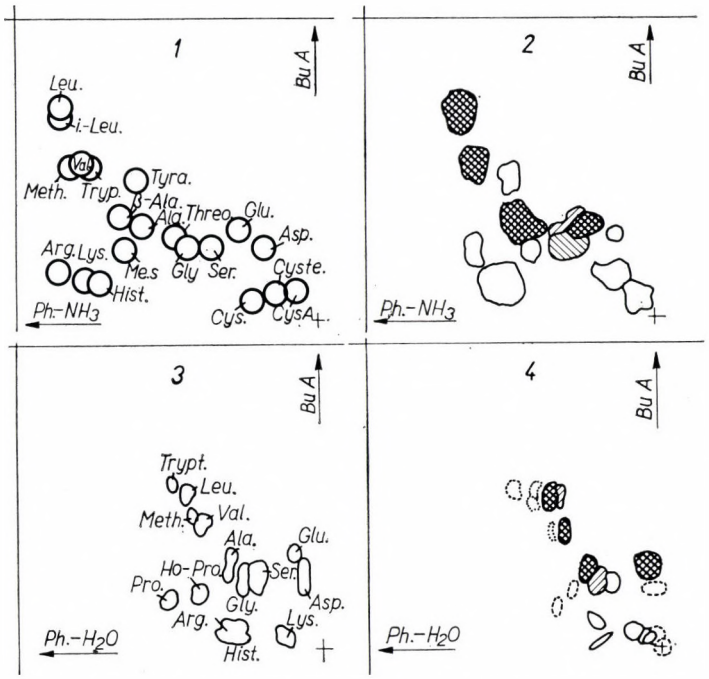

Fig. 10. Schematic drawings of two-dimensional chromatograms

1. Spots of pure amino acids in the two-dimensional paper chromatogram according to SMITH [15]. 2. Two-dimensional paper chromatogram of the supernatant of a spore suspension irradiated with 1000 krad. 3. Two-dimensional thin-layer chromatogram of pure amino acids. 4. Two-dimensional thin-layer chromatogram of the supernatant of a spore suspension irradiated with 1000 krad

typical thin-layer chromatogram. The R_f values belonging to the ninhydrin-positive spots are summarized in Table III.

The supernatants of spore suspensions irradiated with 1000 krad were also investigated with two-dimensional chromatography (Fig. 10).

Chromatography revealed the presence of the following amino acids in the supernatants of irradiated spore suspensions: (iso)leucine, lysine, aspartic acid, cystine and cysteine, threonine, valine, histidine, arginine, tyrosine, serine, methionine, tryptophan. This sequence approximately corresponds to the decreasing order of intensity of the ninhydrin spots.

The amount of ninhydrin-positive compounds directly detectable in the supernatants was also determined. The intensity of the colour produced by the ninhydrin reaction in the supernatants of irradiated suspensions was compared with that occurring in the supernatants of heat-treated (100°C, 30 minutes) suspensions. The quotient thus obtained is shown in Fig. 11 as a function of the radiation dose.

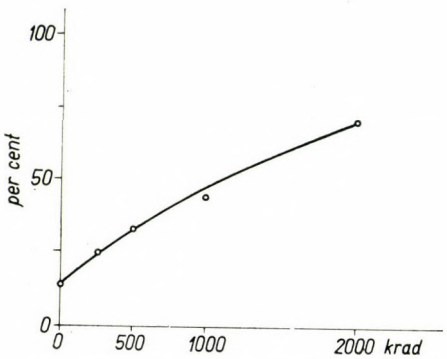

Fig. 11. Concentration of ninhydrin-positive substances as a function of increasing radiation doses in supernatants of spore suspensions.

$$\text{per cent} = \frac{\text{E(irradiated suspension)}}{\text{E(digested suspension)}} \cdot 100$$

Discussion

It is evident from the results that "pseudo-germination", as a direct effect of high radiation doses is related to the increased permeability of the cell walls. Similar conclusions have been drawn by BLACK and GERHARDT [1] concerning the effect of heat treatment.

The substances leaking from the cell under the effect of radiation correspond to the exudate characteristic of physiological germination [7, 8]. The phenomena accompanying irradiation resemble in many respects those observable during mechanical germination of spores [11].

The present findings agree with RIEMANN's hypothesis [10] concerning the conclusive role of Ca-dipicolinate in the development of radiation resistance, as well as in the mechanism of spore formation, and germination. It appears that a higher radiation dose is required to increase permeability than to desorganize cell division. In this respect the effects of irradiation and heat treatment are similar; in both cases there is a time lag between the appearance of DPA in the environment and the death of spores.

In the supernatants of irradiated spore suspensions a number of ninhydrin-positive compounds was observable. Thus, in this respect, too, the direct effect of high radiation doses resembles that of mechanical injury.

A comparison of Fig. 4 and 11 shows that the colour intensity of the ninhydrin reaction in the supernatant increases at a higher rate as a function of irradiation dosage than the amount of Ca-DPA.

According to the earlier literature, resting spores do not contain free amino acids. In a recent paper [19] it has, however, been suggested that dipicolinic acid, while bound to calcium, probably forms at the same time a complex with proteins, *i.e.* amino acids. It may be presumed that as an effect of irradiation the chelate breaks down and the leakage of free amino acids precedes that of dipicolinic acid. It is interesting to note that after lethal heat treatment (90°C, 100 minutes) very few ninhydrin-positive compounds appeared in the amino acid chromatogram of the spores as compared with the chromatogram of irradiated spores.

From the comparison of the amino acid chromatograms of vegetative spores on the one hand and of resting spores, on the other, it appears that in the supernatants of irradiated vegetative spore suspensions the proportion of amino acids (methionine, tyrosine, valine, leucine) possessing R_f values above 0.4 in the applied solvent system exceeds that of the amino acids below 0.4, as compared with the amino acid distribution in the supernatants of vegetative cells. No other difference of consequence could be observed in the amino acid composition of the supernatants of the two cell states.

It has been established that the resistance of resting *B. cereus* cells rapidly decreased to the sensitivity level of vegetative cells, when they were induced to germinate. An obvious way of lowering the resistance to irradiation would therefore be to induce germination by chemical or physical means in resting spores. Since spore germination is a rapid process taking about 15 minutes under the present experimental conditions, this method seems suitable for practical use. Our next aim is to investigate the ability of various compounds to induce germination.

It may be presumed that a lowering of radiation resistance could be achieved also by the application of chemical or physical agents to increase or disorganize cell membrane permeability. Although the change in permeability is not a direct cause of germination, it accompanies the inactivation of factors inhibiting rehydration of resting spores.

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INDUCED LYSOGENESIS OF MYCOBACTERIA

By

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Summary. Lysogenicity has been induced in saprophytic mycobacteria by means of lytic phages. The four lysogenic combinations obtained with *M. friburgensis* and *M. rabinowitsch* and their homologous phages have been examined.

There was no difference between lysogenized and original sensitive strains in colonial morphology and biochemical reactions.

Homologous or related phages had no effect on the lysogenized cultures. The sensitivity of these cultures to other phages changed only slightly or not at all.

Induction in the examined four strains was not observed after ultraviolet irradiation.

Phage adsorption by the four lysogenized strains was influenced by the type of phage as well as the type of the host cell.

Although lysogenicity is a common property of micro-organisms, only four papers have so far been published on the isolation of lysogenic mycobacteria. A rapidly growing lysogenic mycobacterial strain was described by HNATKO [1]. BOWMAN and REDMOND [2] isolated a lysogenic *M. butyricum* culture. SEGAWA *et al.* [3] reported on a non-identified lysogenic mycobacterium strain. Recently, RUSSEL *et al.* [4] isolated a lysogenic *M. fortuitum* strain. No natural lysogens have been found among parasitic mycobacteria, and assumptions of their existence have not been verified [5].

Accidental lysogenization was observed by several authors. The most important of these findings is that of MANKIEWICZ [6]. Artificial lysogenization was reported by TAKEYA *et al.* [7] and RUSSEL *et al.* [8, 9]. The latter authors induced lysogenicity in various mycobacteria including pathogenic strains.

In our experiments lysogenicity was induced in systems containing various combinations of cultures and lytic phages. Two out of seven mycophages isolated in our institute [10] gave serological cross reaction in the homologous antiphage sera (phages friburgensis and rabinowitsch). By the use of these phages and strains *M. friburgensis* and *M. rabinowitsch*, the four possible combinations have been tested. The results are presented in this paper.

Materials and methods

Bacteria and phages. M. friburgensis and *M. rabinowitsch* were obtained from the International Culture Collection Centre, Lausanne. Homologous phages were isolated in our laboratory. The bacteria were cultured for 24 hours on agar slants containing 2 per cent agar and

2 per cent glycerol. Biochemical reactions and phage typing [11] and preparation of stock phages and phage titration [12] were performed as described in previous papers.

Lysogenicity was obtained by means of three different procedures. (1) Plates showing confluent lysis were further incubated, then secondary colonies were streaked on agar plates. (2) As elaborated by LIEB and BERTANI—NICE, and quoted by RUSSEL *et al* [8], 0.01 g/ml $CaCl_2$ was added to a mixture of bacteria and phages (1 : 5). The mixture was incubated at 37°C for 15 minutes then at 0°C for 2 hours. After centrifugation the deposit was resuspended in antiphage serum, incubated at 37°C for 30 minutes, and finally streaked on agar plates. (3) According to our own method, 0.8 ml broth, 0.1 ml phage suspension containing 10^6 particles per ml and 0.1 ml bacterial suspension containing 10 mg cells per ml, were mixed, incubated at 20°C for 48 hours, then streaked onto agar plates.

Demonstration of lysogenization. Five secondary colonies obtained with each of the three methods were subcultured five times. Each subculture was started from single isolated colonies. The presumably pure clones obtained in this way were titrated with the homologous phages. When no phage activity was observed, the strains were treated five times in broth containing antiphage serum in amounts sufficient for the neutralization of 10^5 homologous phage particles within 20 minutes. The 24 hour broth culture of the examined lysogenic strain was dropped on agar plates seeded previously with a 24 hour broth culture of the original (sensitive) strain. The culture was regarded lysogenic when lysis developed at the site of the drop.

Ultraviolet treatment was performed with a germicidal lamp emitting 2537 Å rays in 99 per cent. The cultures were suspended in pH 7, *M*/15 phosphate buffer and exposed to irradiation in a 3—4 mm layer for 60 seconds at a distance of 30 cm. Under the same conditions strain A. T. C. C. 607 was destroyed in 20 per cent.

Examination of phage adsorption and phage production. To 18 ml broth containing 1 mg cells per ml, 2 ml of phage suspension containing 10^5—10^7 particles per ml were added. The system was aerated in a 37°C water bath and sampled at regular intervals. Each 1 ml sample was diluted 1 : 10 with 0°C broth and centrifuged at 5000 r.p.m. for 10 minutes. The supernatant was then titrated in the usual manner. The same method was applied without the addition of phage when spontaneous phage production was examined. Our method differed from that of ADAMS [13] in the use of 1 : 10 instead of 1 : 100 dilutions.

Cell counts. Ten 0.002 ml drops of decimal dilutions of the bacterial suspension were placed on agar plates. The bacterial count was estimated from the number of colonies by TÁRNOK's method [14].

Designation of bacteria and phages. M. friburgensis = Fr, *M. rabinowitsch* = R, phage friburgensis = fr, phage rabinowitsch = r. Lysogenic bacteria are designated by the abbreviation of the strain followed by the abbreviation of the phage in brackets: *M. friburgensis* lysogenized by friburgensis phage = Fr(fr), *M. friburgensis* lysogenized by phage rabinowitsch = Fr(r), *M. rabinowitsch* lysogenized by phage rabinowitsch = R(r), *M. rabinowitsch* lysogenized by phage friburgensis = R(fr).

Results

Two out of seven mycophages isolated in our institute were serologically related and gave cross neutralization reaction with the heterologous antiphage serum. Each of the two sera gave almost identical titres with both homologous and the corresponding heterologous strains.

The lysogenized strains were tested with homologous and heterologous phages. Repeated examinations showed no phage action. The phage resistant strains were then examined as described above. After incubation at 37°C for 24 hours, all strains caused a double ring-type lysis on plates previously seeded with the corresponding original (sensitive) culture (Fig. 1). Between the two lytic zones confluent bacterial growth, within the inner zone a central dense growth was observed. Bacteria transferred from the outer zone of bacterial growth produced double ring-type lysis on plates seeded with sensitive culture. In contrast, bacteria from the inner

central growth gave rise to colonies surrounded by one simple lytic zone. In further studies only the latter type of culture was examined.

A small part of the medium at the site of the lytic zone was transferred into 1 ml broth by means of a capillary. In order to demonstrate that lysis

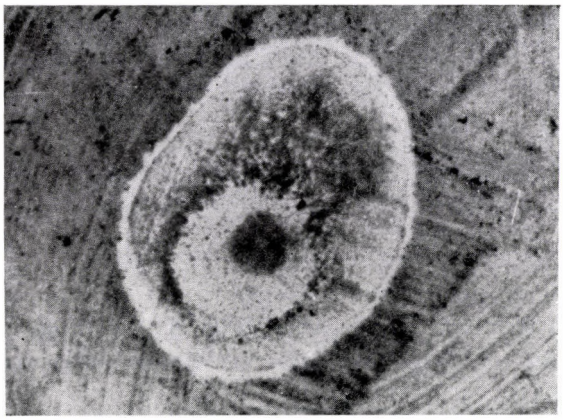

Fig. 1. Effect of lysogenized strain R(r) on sensitive *M. rabinowitsch* culture

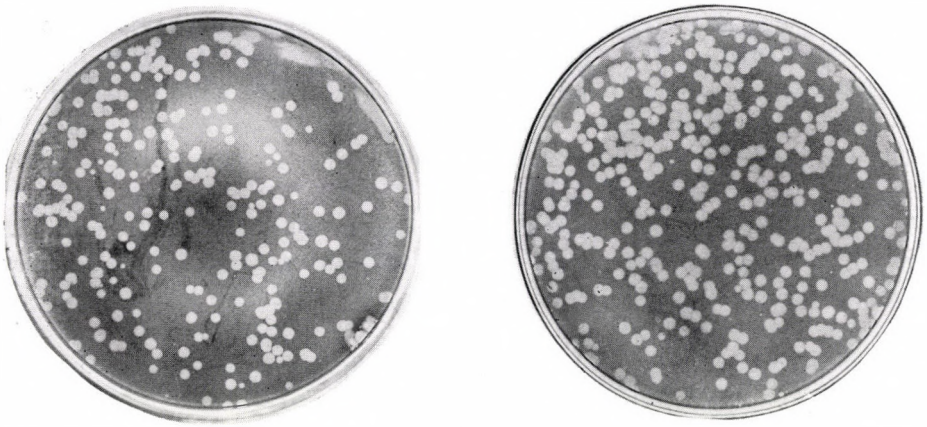

Fig. 2. Plaques produced by lysogenic strain Fr(fr) (A) and by phage friburgensis (B)

was caused by phage action, the broth was titrated with a sensitive strain. As presented in Figs 2A and 2B phage action was always revealed. Phage concentration of the broth was of the order of 10^5. According to our observations the same number of particles is usually present in the average plaque formed by mycobacterial phages.

Table I

Titration of phages produced by lysogenized strains

Phages produced by lysogenized strains	Indicator strains		Lysogenic strains			
	M. friburgensis	*M. rabinowitsch*	Fr(fr)	Fr(r)	R(r)	R(fr)
Fr(fr)	3×10^5	1×10^5	—	—	—	—
Fr(r)	1×10^5	2×10^5	—	—	—	—
R(r)	2×10^5	3×10^5	—	—	—	—
R(fr)	5×10^5	1×10^5	—	—	—	—

Filtrates of 24-hour aerated cultures of the lysogenized strains were titrated with sensitive and lysogenic strains. As shown in Table I, the titres for indicator strains were of the order of 10^5. Phage action on lysogenic strains was not observed.

Sensitive and lysogenic strains were examined for staining and colonial morphology. Qualitative peroxidase, catalase, niacin, nitrate reduction and Bönicke's amide tests were also performed. As shown in Table II, no difference was revealed between the original and lysogenized strains.

The lysogenized strains were compared also by the phage-typing method used in this institute [11]. The results are presented in Table III. In contrast to *M. friburgensis*, lysogenized strains Fr(fr) and Fr(r) were not lysed by friburgensis and rabinowitsch phages. The same held true for strains R(r) and R(fr), which, however, showed some additional differences in comparison with the phage pattern of the original strain.

The lysogenized strains were subjected to ultraviolet treatment. The dose used destroyed cells of strain A.T.C.C. 607 in 20 per cent. Our lysogenized cultures were killed in 75—90 per cent. With the decrease in the number of viable cells, the phage count also decreased. In case of inducibility by irradiation an increased phage count would have been found. The ratio of viable cells and free phages (B/P), however, remained unchanged after irradiation (Table IV).

In order to show whether the lysogenized strains are able to adsorb phages serologically related to the prophage, the following experiments were performed. (1) Phage adsorption and phage production by strains R and Fr. (2) Spontaneous phage production by the four lysogenized cultures. (3) Phage adsorption of homologous and related phages by the four lysogenized cultures. (4) Checking of the titre of phages. The results are shown in Figs 3A, 3B, and 4A, B, C, D. In the graphs the logarithm of phage count is plotted against time.

Table II

Biochemical reactions

Strains	Bönicke's amides										Nitrate reduction	Niacin	Catalase	Peroxidase	Staining, liquid Sula medium, 3 days	Colonies
	Acetamide	Benzamide	Urea	Isonicotinamide	Nicotinamide	Pyrazinamide	Salicylamide	Allantoin	Succinamide	Malonamide						
M. friburgensis	—	+	++	+	+	+	—	—	±	—	pink	—	++++	++++	Some cyanophilic rods, separate dark granules. No important difference among strains	No morphological difference
Fr(fr)	—	++	++	+	++	+	—	—	+	—	pink	—	++++	++++		
Fr(r)	—	+	+	+	+	+	—	—	+	—	pink	—	++++	++++		
M. rabinovitsch	—	++	++	+	+	+	—	—	±	—	pink	—	++++	++++		
R(r)	—	+	++	+	++	+	—	—	+	—	pink	—	++++	++++		
R(fr)	—	+	++	+	+	+	—	—	±	—	pink	—	++++	++++		

Table III

Phage sensitivity

Strains	Phages															
	phlei	pellegrini	minetti	smegmatis	butyricum	friburgensis	rabinowitsch	"polanus" (Macander)	D_{29} (Froman)	$MyF_1P/58$ (Sula)	$MyF_2P/59$ (Sula)	$MyF_3P/59$ (Sula)	lacticola (Penso)	∅ 101 A (Redmond)	DS6A (Redmond)	Vaccae B_5 (Bönicke)
M. friburgensis	—	—	—	3	3	4	4	3	4	4	4	4	4	4	—	—
Fr(fr)	—	—	—	3	3	—	—	4	4	4	4	4	4	4	—	—
Fr(r)	—	—	—	3	3	—	—	4	4	3	4	4	4	4	—	—
M. rabinowitsch	—	—	—	2	—	4	4	3	—	3	2	2	4	4	—	—
R(r)	—	—	—	—	—	—	—	3	—	3	—	—	4	4	—	—
R(fr)	—	—	—	3	3	—	—	3	—	3	—	—	4	4	—	—

Key: 4 = confluent lysis, 3 = semiconfluent lysis, 2 = more than 10 separate plaques, 1 = 1—10 plaques, — = no lysis.

Table IV

Effect of ultraviolet irradiation on lysogenic strains

Strains	Irradiation	Bacterial count	Percentage decrease	Phage count	B/P ratio
Fr(fr)	—	1.6×10^7	87.5	3×10^5	$1 : 1.9 \times 10^{-2}$
	+	2×10^6		4×10^4	$1 : 2 \times 10^{-2}$
Fr(r)	—	1.6×10^7	87.5	6×10^5	$1 : 3.7 \times 10^{-2}$
	+	1.8×10^6		7×10^4	$1 : 3.8 \times 10^{-2}$
R(r)	—	1.4×10^7	75.0	1×10^6	$1 : 7.1 \times 10^2$
	+	3.5×10^6		2.5×10^5	$1 : 7.1 \times 10^{-2}$
R(fr)	—	1.4×10^7	90.0	3×10^6	$1 : 2.1 \times 10^{-2}$
	+	1.4×10^6		3×10^5	$1 : 2.1 \times 10^{-2}$

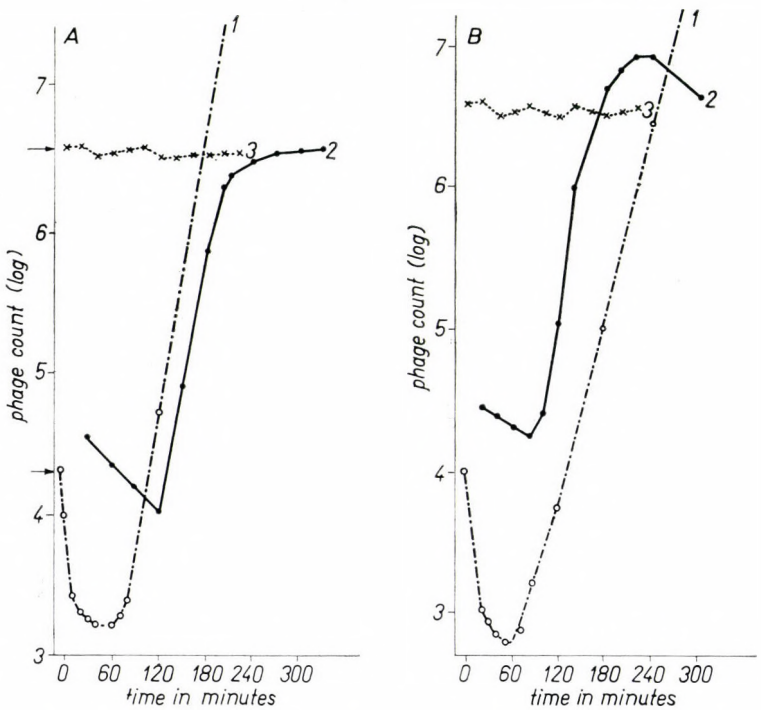

Fig. 3A. Phage adsorption and phage production by *M. friburgensis* infected with friburgensis phage (1). Spontaneous phage production by lysogenic strain Fr(fr) (2). Bacterium-free control of friburgensis phage (3)

Fig. 3B. Phage adsorption and phage production by *M. rabinowitsch* infected with rabinowitsch phage (1). Spontaneous phage production by lysogenic strain R(r) (2). Bacterium-free control of rabinowitsch phage (3)

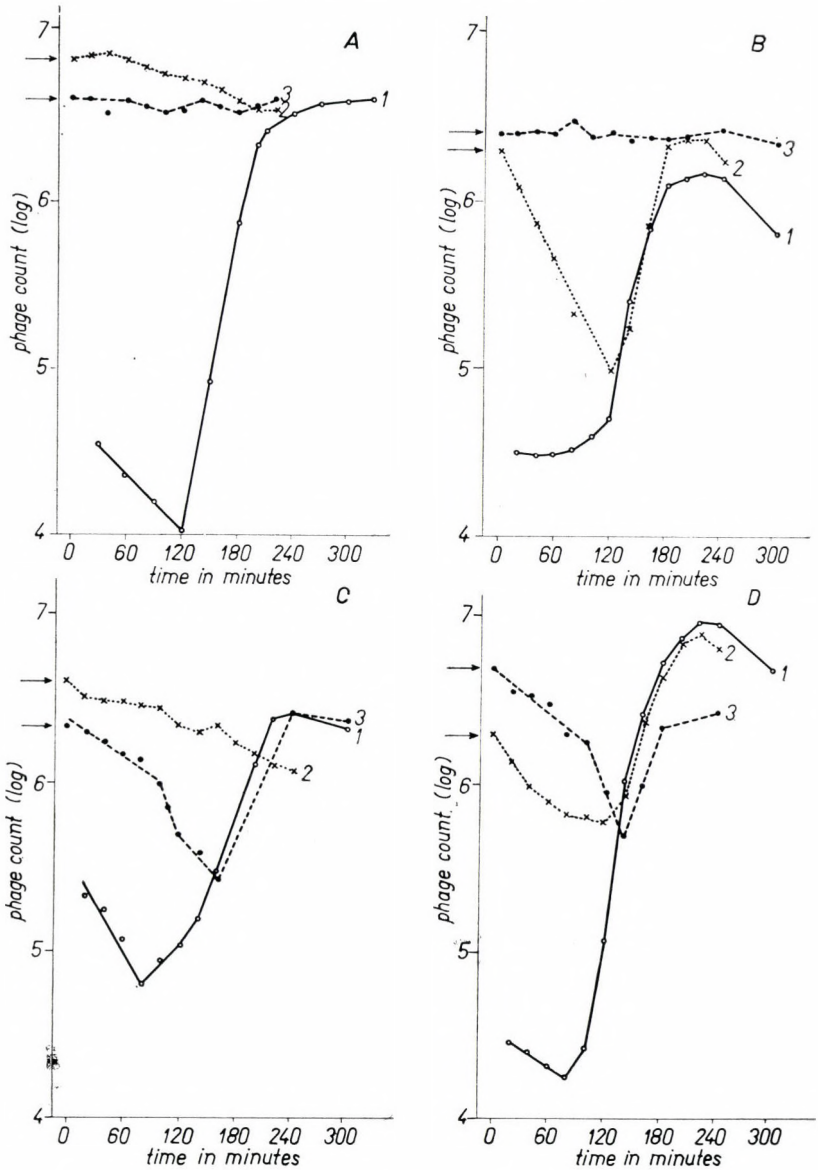

Fig. 4. Spontaneous phage production (1) and adsorption of homologous (2) and related (3) phages. A = Fr(fr), B = Fr(r), C = R(fr), D = R(r)

Discussion

In the present study lysogenicity has been induced in saprophytic mycobacteria by phage treatment. Clone purity was secured by five consecutive subcultures of the treated bacteria by the single colony technique. To exclude

the possibility that the cultures were phage carriers, five consecutive passages were performed in a medium containing antiphage serum neutralizing 10^5 particles per ml. The new culture was considered lysogenic when it produced phages acting on the original sensitive strain and was resistant to the lytic activity of the homologous phage.

No cultural or biochemical differences were revealed between the lysogenized and original strains. RUSSEL *et al.* [8], who performed cross neutralization and animal experiments in addition to some biochemical tests, observed no appreciabe difference between the original (sensitive) and lysogenized mycobacterial cultures.

Our lysogenized strains were examined with the routine phage-typing method employed in this institute. Development of lysogenicity eliminated the lytic action of homologous and serologically related phages. In sensitivity to other phages only minimal changes were observed.

Induction of phage production in the lysogenized cultures was attempted by means of ultraviolet irradiation. The employed dose destroyed lysogenic cells in 75—90 per cent. Parallel with the decrease of viable count, there was a decrease in phage count. According to NORTHROP [15], under defined cultural conditions the ratio of lysogenic cells and produced phages is a constant value about the order of 10^{-2}. In our experiments the B/P ratio was unchanged after ultraviolet treatment. It has therefore been concluded that irradiation fails to cause induction in our cultures.

Results of testing the adsorption by lysogenized strains of homologous and related phages are summarized in Table V. It is seen that phage friburgensis hindered the adsorption of both homologous and related phages. Phage rabinowitsch exerted no influence on the adsorption of either of them. *M. friburgensis* in the lysogenic state, independently of the phage used for lysogenization, did not adsorb the homologous phage. The antagonistic activity of friburgensis phage against the homologous phage (r) was eliminated by *M. rabinowitsch*.

Table V

Adsorption experiments

Strain	Phage	
	friburgensis	rabinowitsch
Fr(fr)	—	—
Fr(r)	—	+
R(fr)	—	+
R(r)	+	+

The conclusion has been drawn that adsorption in the examined lysogenic systems is influenced not only by the phage but also by the type of the host cell.

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ADENOVIRUS TYPE 7 OUTBREAK IN A KINDERGARTEN

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Summary. In January, 1964, an outbreak of upper respiratory disease was observed in a kindergarten in a peripheral district of Budapest. Out of a total of 36 children 26 contracted the disease. Virus isolation was attempted from both nasal and throat swabs obtained from 22 children. Samples from 14 patients yielded adenovirus type 7. In one case the simultaneously obtained nose and throat swabs yielded type 7 and type 3 adenoviruses, respectively. A total of 19 paired sera were examined by both CF and HI tests using type 3 and type 7 adenoviruses as antigens. The HI test proved to be the more sensitive one. Using the latter method the aetiological role of type 7 adenovirus could be proved in 17 cases. The CF test yielded positive results in 9 cases only.

Since the first description of adenoviruses [1] a number of authors has stressed the aetiological importance of these agents, paticularly in diseases of the upper respiratory tract. Type 7 has been known to belong to the so-called "epidemic" adenoviruses. Its isolation in non-epidemic periods is rarely successful. Epidemics caused by the virus have been observed among military recruits [2, 3] and schoolchildren [4], and also in day-schools and nurseries [5—15].

The present paper is a report on an epidemic of type 7 adenovirus infections started at the beginning of January, 1964, in a nursery situated in a peripheral district of Budapest. Out of a total of 36 infants 26 developed the disease within a two-week period. The main symptoms were fever, mild conjunctivitis, swelling of the submandibular lymph nodes, serous rhinitis and pharingitis. In nine cases each bronchitis and pneumonia associated themselves to the primary upper respiratory tract disease. The average duration of illness was between 7 to 14 days. To clarify the aetiology, an attempt was made to isolate the agent from 22 patients and serological studies were performed on paired sera of 19 children. The results obtained have proven the aetiological role of adenovirus type 7. A detailed report on the epidemic and on the performed virological studies is given below.

Materials and methods

Sampling. Nasal and throat swabs were taken simultaneously (Jan. 17) from all the 22 children. The swabs were rinsed thoroughly into PARKER's medium 199 (with antibiotics) in separate test tubes each. The fluid then was centrifuged for 20 minutes at 8,000 r.p.m. To the supernatant, 2,000 U of penicillin, 2 mg of streptomycin and 100 U of Mycostatin per ml were added and the material was stored at 4°C until used.

The first blood samples were taken simultaneously with the swabs, the second ones 14 days later. The sera were inactivated at 56°C for 30 minutes.

Tissue cultures. Three parallel tubes of both primary monkey kidney (MK) and HeLa cells were inoculated each with 0.1 ml of the samples. The maintenance media for MK and HeLa cultures were PARKER's medium 199, and Hanks' medium enriched with 0.5 per cent lactalbumine-hydrolyzate and 10 per cent rabbit serum, respectively.

Identification. About 100 CPD_{50} doses of the isolates were examined in the neutralization test on HeLa cells using adeno type 1—8, 11, and 14—17 specific immune sera prepared by the Virus Department of the State Institute of Hygiene, Budapest [16]. The virus-serum mixtures were incubated for 2 hours at 37°C.

Serological studies. The paired sera of 19 patients were examined by complement fixation (CF) and haemagglutination-inhibition (HI) tests using the micromethod of TAKÁTSY [17]. Non specific inhibitors were removed from the sera by adsorption with 20 per cent kaolin and 2 per cent vervet monkey red blood cells [18]. Titrations were performed in twofold dilution series of sera against 4 haemagglutinating units each of types 3 and 7 of adenoviruses. The virus-serum mixtures were incubated at room temperature for 1 hour. This was followed by the addition of 1 per cent vervet monkey red blood cells. The results were read after an hour of incubation at 37°C.

Results

Out of the total of 44 samples obtained from 22 patients, 20 yielded cytopathogenic (CP) agents. On identification 19 agents turned out to be type 7 while the remaining 1 was type 3 of adenovirus. Results of the isolations and serological examinations are given in Table I.

Virus was isolated in 14 out of the 22 patients (63.6 per cent). Both the nasal and the throat swab was positive in 27.2 per cent. The incidence of

Fig. 1. Serological responses to adenovirus types 7 and 3, in CF and HI tests

Table I

Results of virological examinations

No.	Initials and age (years) of patients.	Clinical picture	Adenovirus type isolated from		Serum sample	Titres* of sera in		
			nasal secretion	pharyngeal secretion		CF	HI ad 7	HI ad 3
1	B. I. 6	URI	7	7	I	8	—	—
					II	16	32	—
2	H. J. 3	PN	7	7	I	8	128	256
					II	16	256	256
3	V. K. 6	PN	7	7				
4	R. K. 5	PN	7	7	I	—	—	—
					II	4	16	—
5	N. J. 5	BR	7	7	I	64	8	—
					II	64	32	—
6	P. K. 7	BR	7	3	I	128	—	—
					II	32	16	32
7	P. M. 7	URI	7	—	I	32	—	—
					II	128	32	—
8	C. J. 5	PN	7	—	I	—	—	—
					II	8	16	—
9	F. P. 4	BR	7	—	I	—	64	256
					II	—	256	256
10	L. O. 5	PN	7	—	I	8	32	64
					II	128	128	256
11	R. F. 6	BR	7	—	I	8	—	—
					II	32	16	—
12	T. L. 5	URI	—	7	I	16	—	—
					II	16	16	—
13	H. A. 5	PN	—	7	I	8	—	—
					II	32	—	—
14	S. Á. 5	BR	—	7	I	4	64	256
					II	64	64	256
15	P. P. 6	URI	—	—	I	—	32	64
					II	4	256	32
16	J. A. 3	PN	—	—	I	8	—	—
					II	8	32	—
17	R. K. 3	PN	—	—	I	—	—	—
					II	—	32	—
18	B. Z. 5	PN	—	—				
19	T. K. 5	BR	—	—	I	16	16	16
					II	16	32	16
20	J. I. 6	BR	—	—	I	32	16	16
					II	4	32	16
21	P. Z. 3	BR	—	—	I	4	—	—
					II	8	—	—
22	P. I. 5	BR	—	—				

URI = upper respiratory illness
BR = bronchitis
PN = pneumonia
* = reciprocals
— = negative

positivity in a single sample, was 22.7 and 13.6 per cent for nasal and throat swabs, respectively. Patient No. 6 harboured type 7 adenovirus in his nose and type 3 adenovirus in his throat. This finding was verified by repeated isolations from the two simultaneously taken samples.

In the serological tests all paired sera exhibiting a fourfold or twofold titre increase or a persisting titre of 1 : 32 or higher were regarded as positive. Using type 7 adenovirus the CF and HI were positive in the sera of 9 and 17 patients, respectively. With type 3 adenovirus, however, only 5 patients exhibited a positive serological reaction (Fig. 1).

Fourfold or higher increase of HI antibody titres, *i.e.* a proof of acute infection, was most frequent against type 7 of adenovirus. Thus, both the isolation experiments and the serological studies yielded evidence of the aetiological role of adenovirus type 7 in the observed outbreak.

Discussion

Nasal and throat swabs were collected at the same time from all children in the kindergarten. Thus, in several cases the optimal point of time of sampling was missed. In some cases even the date of the onset of illness was unknown. Nevertheless, the incidence of positive isolation results was quite high. It is worthy of note that in the early phase of the disease the nasal swabs while in the late phase the throat swabs exhibited a higher incidence of positivity. The possibility has arisen of a type 7 carriership similar to that observed with other adenoviruses and supported by the observation of PEREIRA and KELLY [19] who succeeded in recovering type 7 adenovirus from a child's adenoid four months after the acute illness.

HeLa cell cultures proved to be superior to MK for isolation purposes. Seventeen out of the total of 20 CP agents isolated were, however, recovered from MK cells, too. Nevertheless, in HeLa cells the virus attained titres of 10^{-2} to 10^{-3}, while in MK only the undiluted material proved to be active.

In neutralization tests ROWE *et al* [20] found a cross reaction between types 3 and 7 of adenoviruses. No such phenomenon was observable with the strains isolated by us. Except for the single type 3 strain, none of the CP agents isolated were neutralized by immune sera other than anti-type 7.

The outbreak was a mild one. The pneumonias observed were not severe except for one case. The latter required treatment for more than one month. Virus was isolated from 6 out of the 9 pneumonia cases, from 5 out of the 9 bronchitis cases, and from 3 out of the 4 patients with upper respiratory disease. Thus our results support the possible aetiological importance of type 7 adenovirus. This agent may produce banal upper respiratory symptoms with occasional spreading into the lower parts of the respiratory tract. The outbreak reported

by us resembled that observed in 1959 in Stockholm [4], except that gastrointenstinal symptoms were not present in our patients. The occurrence of severe, sometimes fatal pneumonias seems to be restricted to children (particularly babies) hospitalized or living under poor hygienic conditions.

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STUDIES ON THE FERMENTATION OF STEREUM PURPUREUM

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Summary. It has been shown that fermentation of *Stereum purpureum* meets industrial requirements. By use of adequate technology homogeneously dispersed cultures can be prepared. Formation of pellets depends on the ingredients and the pH of the medium. Pigment production varies with the media used for subculturing the strain and with the pH of the fermentation medium.

In the last decades fermentation industry has undergone a rapid development. At the beginning the motive force of this progress was the large-scale production of penicillin. Today a wide variety of antibiotics is produced by industrial fermentation. In addition to bacteria and actinomycetes, Fungi Imperfecti are mainly employed and experiments with basidiomycetes are in progress. HUMFELD [2, 3], HUMFELD and SUGIHARA [8, 9], SUGIHARA and HUMFELD [7], BLOCK *et al.* [1, 10], SANTORO and CASIDA [5] and ESPENSHADE [4] used aerated fermentation for growing the mycelium of various basidiomycetes. The main purpose of these experiments was to obtain mycelium for the preparation of human or animal foods.

Submerged culturing of basidiomycetes would be a great step in the development of fermentation industry, provided that the mycelium obtained in this manner contains substances present in the reproduction elements of different fungi. The purpose of the present experiments was to examine the growth of a wood-rotting organism, *Stereum purpureum* in pure aerated submerged cultures. The reproducibility of homogeneous (macroscopically evenly dispersed) submerged cultures has also been studied.

Materials and methods

Experiment 1. Solid medium Sp—1 was prepared as follows: 0.83% corn steep liquor (50% dry weight), 1.5% potato starch, 0.2% potassium dihydrophosphate, 0.4% ammonium sulphate and 0.3% calcium carbonate were dissolved by boiling. After the addition of 3% agar the medium was distributed at 150 ml amounts into Roux bottles. Sterilization was performed at 120°C for 40 minutes. The bottles were inoculated with cultures grown on malt extract agar. Incubation lasted for 14 days at 30°C. Fresh bottles were inoculated with agar pieces 2 cm^2 in size carrying the surface mycelium culture. After two weeks' incubation a third subculture was prepared.

Experiment 2. Liquid Sp—1 medium contained, with the exception of agar, the same ingredients as the solid Sp—1 medium. The medium was distributed at 100 ml portions into

500 ml Erlenmeyer flasks then sterilized at 120°C for 60 minutes. The flasks were inoculated with surface cultures as in Experiment 1, then shaken for 7 days at 30°C. Three parallel cultures were examined.

Experiment 3. Preparation of medium Cs—60: An aqueous suspension containing 2% soy flour and 0.3% sodium chloride was adjusted to pH 6.0. After boiling for 5 minutes, the suspension was filtered through cotton wool. To the soy extract 2% glucose, 0.4% ammonium chloride, 0.2% potassium chloride, 0.01% manganese sulphate, 0.04 mg/100 ml of cobalt nitrate and 0.05 mg/100 ml of boric acid were added. The boiled medium was distributed at 100 ml portions into 500 ml Erlenmeyer flasks. The content of each flask was adjusted to different pH values: 7.0, 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0. The flasks were then sterilized at 120°C for 40 minutes.

Preparation of medium Z/8: 1% soy flour, 4% potato starch, 0.5% calcium carbonate, 0.4% ammonium chloride, 0.3% sodium chloride, 0.2% potassium chloride, 0.01% manganese sulphate, 0.04 mg/100 ml of cobalt nitrate, 0.05 mg/100 ml of boric acid and 0.5% palm oil. After boiling the pH was adjusted to 7.0, then the medium was distributed at 100 ml amounts into 500 ml Erlenmeyer flasks and sterilized at 120°C for 60 minutes.

Three parallel flasks with media Cs—60 and Z/8 were seeded with 2 ml aliquots of homogeneously dispersed cultures grown at pH 3.5 in Experiment 2. The flasks were shaken for 7 days at 30°C.

Experiments 4 *to* 7. By use of medium Z/8, the experiments were performed as described in Experiment 3. The flasks were always inoculated with the homogeneously dispersed culture obtained in the previous experiment.

Submerged cultures were aerated by shaking at 260 cycles per minute through a distance of 2 cm.

Sterility of the cultures was checked daily by microscopic examination and culturing in broth. Sterility testing of surface cultures was performed by a similar method using the condensation water of the bottles.

Macroscopic properties of the mycelium were examined in surface cultures. Therefore in every experiment solid Sp—1 media were also seeded.

Homogeneity (dispersed or containing pellets) and colour of submerged cultures were evaluated visually.

Results

The results are summarized in Table I.

Experiment 1. Surface cultures yielded in 14 days abundant mycelium extending evenly over the whole surface of the medium. The culture had a loose, cotton surface, rusty red in colour (Paragraph 1 in Table I).

Experiment 2. In liquid Sp—1 media at initial pH values of 5.0, 4.5, 4.0 and 3.5, giant light yellow pellets were produced. At pH 3 a paprika-red dense homogeneous culture was obtained (Paragraph 2 in Table I).

Experiment 3. In glucose-containing pH 7.0 and 5.0 Cs-60 medium the mycelium formed one giant pellet. At other pH values the culture remained homogeneous in this medium. In starch-containing medium Z/8 homogeneous cultures were obtained. The colour of the fermentation fluid varied from colourless through light red to deep orange according to the decrease in the initial pH values. The colour was independent of the culture's dispersity. Surface cultures were similar to those described in Experiment 1 (Paragraph 3 in Table I).

Experiment 4. In all flasks homogeneous cultures were obtained. Orange pigment was produced only in cultures seeded with pH 4.0 and 4.5 cultures of the previous experiment. Other cultures remained colourless. No alteration was observed in surface cultures (Paragraph 4 in Table I).

Table I

Dispersity of Stereum purpureum cultures following passages

Stereum purpureum
(agar slant culture)

1.	Sp-1 medium surface culture							
2. pH Shaken culture	5.0 Sp-1 Y, p	4.5 Sp-1 Y, p	4.0 Sp-1 Y, p	3.5 Sp-1 Y, p	3.0 Sp-1 R, h			
3. pH Shaken culture	7.0 Cs-60 CL, p	6.5 Cs-60 LR, h	6.0 Cs-60 LR, h	5.5 Cs-60 LR, h	5.0 Cs-60 O, p	4.5 Cs-60 O, h	4.0 Cs-60 O, h	7.0 Z/8 CL, h
4. pH Shaken culture		7.0 Z/8 CL, h	7.0 Z/8 CL, h	7.0 Z/8 CL, h		7.0 Z/8 O, h	7.0 Z/8 O, h	7.0 Z/8 CL, h
5. pH Shaken culture		7.0 Z/8 CL, h	7.0 Z/8 CL, h	7.0 Z/8 CL, h		7.0 Z/8 O, h	7.0 Z/8 O, h	7.0 Z/8 CL, h
6. pH Shaken culture		7.0 Z/8 CL, h	7.0 Z/8 CL, h	7.0 Z/8 CL, h		7.0 Z/8 O, h	7.0 Z/8 O, h	7.0 Z/8 CL, h
7. pH Shaken culture		7.0 Z/8 CL,h	7.0 Z/8 CL, h	7.0 Z/8 CL, h		7.0 Z/8 O, h	7.0 Z/8 O, h	7i0 Z/8 CL, h
8. pH Shaken culture		7.0 Z/8 CL, h	7.0 Z/8 CL, h	7.0 Z/8 CL, h		7.0 Z/8 O, h		7.0 Z/8 CL, h

p = pellet formation, h = homogeneous (disperse) culture, Y = yellow, R = red LR = light red, O = orange, CL = colourless

Experiment 5. The results were identical with those obtained in Experiment 4 (Paragraph 5 in Table I).

Experiment 6. The results were identical with those obtained in Experiment 5 (Paragraph 6 in Table I).

Experiment 7. No changes were observed. The 4th Z/8 subculture originating from pH 4.0 culture was contaminated by bacteria; therefore the results could not be evaluated (Paragraph 8 in Table I).

Discussion

The aspects of industrial fermentation of *Stereum purpureum* have been examined. The experiments were extended to the production of homogeneous cultures and reproducibility of the results.

From Experiment 2 it may be concluded that a homogeneous culture can be prepared in corn-steep starch medium (Sp-1) at pH 3.0. This culture was strongly pigmented and retained its original properties throughout 5 subcultures in different media. Homogeneous cultures were obtained at pH ranges 4.0—6.5 in glucose medium (Cs-60) used in Experiment 3. At some pH values (5.5—6.5), however, pigmentation was weak. Starch and soy-containing medium Z/8 at pH 7 (Experiments 4—7) was adequate for the preservation of the previously developed main characters (dispersity, pigment production) of the mycelium in serial subcultures.

Production of homogeneous cultures is an important industrial requirement, as intensive metabolism takes place only in such cultures. Reproducibility of the results is also important in this respect. From our experiments it may be concluded that the formation of pellets, in addition to its dependence on the initial pH, is associated with the order in which various media are used for consecutive subcultures. The influence of the glucose and starch content of the medium is also evident. Investigations into the effect of amylase activity will be reported in a subsequent paper.

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AMYLASE ACTIVITY OF SUBMERGED STEREUM PURPUREUM CULTURES

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Summary. The amylase activity of homogeneous aerated cultures of *Stereum purpureum* has been examined.

(i) The starch content and pH of starch-soy extract medium changes during sterilization.

(ii) Homogeneous submerged cultures of *S. purpureum* decompose starch rapidly.

(iii) Inhibition of amylase activity is proportional to the amount of a decomposition product formed during sterilization of the medium.

(iv) Maximum amylase activity is exerted in starch-soy extract medium adjusted to pH 4 before sterilization.

(v) The fermentation fluid of *Stereum purpureum* probably contains an enzyme breaking up 1,6-glucoside bonds.

In recent years several authors have studied the problems of basidiomycetes fermentation [1—5]. In a previous paper [8] we have reported on the cultivation of *Stereum purpureum*. It was noted that the result of aerated submerged fermentation is influenced by the starch content of the medium.

The present paper deals with the amylase activity of *Stereum purpureum* with special regard to its association with the sterilization process.

Materials and methods

Inoculum. A homogeneous vegetative culture of *Stereum purpureum* was prepared with solid Sp—1 and liquid Cs—60 and Z/8 media, as described previously [8]. The fermentation fluid was distributed at 10 ml portions into test tubes and stored at 2°C for not more than 30 days. Fermentation media were seeded with the content of these tubes.

Fermentation. Experiment 1: medium Z/8 was used. Experiment 2: aliquots of medium Z/8 were adjusted with sulphuric acid to pH 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0. The media were distributed at 100 ml amounts into 500 ml Erlenmeyer flasks and sterilized at 120° C for 60 min utes. Each flask was seeded with the content of one inoculum tube. Incubation was performed by shaking at 30°C.

Determination of amylase activity. In Experiment 1 the modified method of SMITH and ROE [6] was used. This method involves the addition of known amounts of starch to the enzyme solution and the determination of the residual substrate. The method was modified in that pH 6.0 instead of pH 7.2 phosphate buffer was used. The filtered fermentation fluid was applied as an enzyme solution.

In Experiment 2 the indirect method described by FIELDS *et al.* [7] was used for amylase determination.

Determination of starch concentration. In Experiment 2 the starch content of fermentation media before and after sterilization was determined as follows. One g of starch was boiled in 50 ml of water for 5 minutes, then the volume was made up to 100.0 ml (10,000 μg starch per ml). From the stock solution various dilutions ranging from 10 to 100 μg/ml, were prepared. To 10

ml aliquots of each dilution 0.5 ml of potassium iodide-iodine reagent was added. Ten ml of water plus 0.5 ml of reagent was used as a blank solution. Optical densities were read in the Uvifort photometer at 578 mμ; blank reading was set at zero. From the results a standard curve was drawn. The medium or fermentation fluid was so diluted as to contain 10 to 100 μg of starch per ml, then read in the photometer. The starch content was calculated from the standard graph. The limits of error were $\pm$ 4 per cent.

Determination of pH was performed by means of an electric pH meter.

Each 100 ml portion of the fermentation medium was seeded with 10 ml of inoculum culture stored at 2°C. Four parallel cultures were examined.

Checking of fermentation and sterility was performed as described previously [8].

For amylase activity assay the fermentation fluids were sampled at 4 hour intervals.

In Experiment 2 the pH of the media was determined before and after sterilization. Starch content was determined after sterilization.

Results

Amylase activities (mg/ml) obtained in Experiment 1 with SMITH and ROE's method are presented in Table I.

It is seen that negative and positive values varied in the consecutive samples. The results were similarly irregular in repeated experiments.

The percentage of starch content of the media used in Experiment 2 before and after sterilization and of starch decomposed during sterilization,

Table I

Amylase activity of Stereum purpureum

Time of incubation, hours	Activity (decomposition of mg starch/ml)
4	— 0.95
8	— 2.30
12	— 1.40
16	0.00
20	+ 8.00
24	+12.22
28	+18.60
32	+19.80
36	+ 8.90
40	— 1.20
44	+ 3.40
48	+ 3.00

are shown in Fig. 1. The graphs under the columns indicate the corresponding pH values before and after sterilization. It is evident that the degree of starch decomposition varied with, but was not proportional to, the initial pH. The

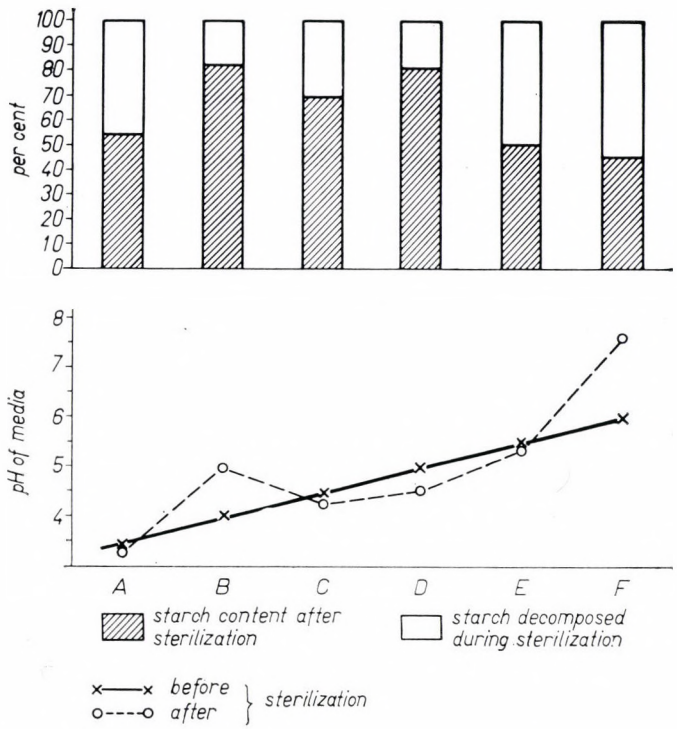

Fig. 1. Alteration of starch content and pH of media during sterilization

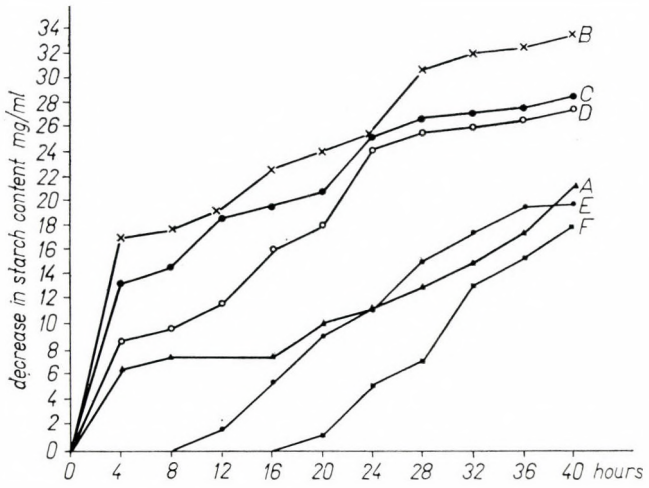

Fig. 2. Amylase activity in media sterilized on various pH

amount of decomposed starch was characteristic of the corresponding pH value. Minimum starch decomposition was obtained at initial pH 4.0.

Fig. 2 shows the results obtained in the media presented in Fig. 1. Amylase activity is expressed as the mg/ml decrease in strach content of the medium [7].

The amylase activity curves can be divided into 3 groups. Group 1 includes curves B, C and D. These cultures exerted a high activity in the first 4 hours of fermentation and decomposed 51, 43 and 29 per cent of the post-sterilization starch content, respectively.

Group 2 is represented by curve A. These cultures decomposed 30 per cent of the starch content within the first 4 hours; on further incubation they were inactive. Later their activity increased again, but remained lower than that exerted by Group 1 cultures.

Group 3 includes cultures E and F, which did not decompose starch in the first 8 or 16 hours.

Discussion

The previous paper [8] presented results obtained with homogeneous submerged starch-soy medium cultures of *Stereum purpureum*. These cultures grew abundantly and produced no pellets. It has therefore been concluded that in addition to the pH, the absence of pellet formation is associated with the starch content of the medium, or the amylase activity of the culture. From amylase activity determinations the following conclusions have been drawn.

The amylase activities measured in Experiment 1 seem to indicate that in some incubation periods the organism was synthesizing starch. In reality the negative results were attributable to the amylopectin content of the water-soluble starch preparation used in the experiments. Amylase-like side chains liberated from amylopectin after the breaking up of 1,6-glucoside bonds increase the intensity of the potassium iodide-iodine colour reaction. On the other hand, free amylase molecules containing 1.4-glucoside bonds are decomposed by amylase, therefore the number of amylase molecules and consequently the intensity of colour reaction decreases. Accordingly, the method of SMITH and ROE [6] measures the resultant of the breaking up of 1,4- and 1,6-glucoside bonds, thus for the lack of a suitable substrate the method evidently fails to yield reliable results for amylase activity.

According to the method of FIELDS *et al.* [7], amylase activity is expressed as the decrease in the starch content of the medium. In Experiment 2 this method was used.

Sterilization caused changes in the pH and the starch content of the media. Comparison of post-sterilization pH and unattacked starch content revealed that decomposition was least at pH 4.0. From the irregular association

of the pH and the degree of starch decomposition it may be concluded that the pH is altered during sterilization by the hydrolysis of soy flour.

Amylase activity graphs uniformly indicate that at the beginning of fermentation the enzyme added to the medium with the inoculum is inhibited proportionally to the concentration of decomposition products formed on heating of the medium. In one type of amylase curves a high activity is evident from the moment of seeding (Group 1). Media seeded with these cultures contained the highest concentration of starch and the lowest amounts of decomposition products.

In cultures falling into Group 2 larger amounts of decomposition products seemed only to hinder the synthesis of new amylase. In group 3 the inoculum amylase was inactive or the culture produced no amylase in the first 8 or 16 hours of fermentation.

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EFFECT OF pH ON THE GROWTH IN SUBMERGED CULTURE OF SOME WOOD-ROTTING FUNGI

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Summary. Homogeneous, aerated submerged cultures of *Stereum purpureum*, *Trametes versicolor* and *Phellinus igniarius* were found to utilize glucose. Higher mycelium yields were obtained in soy-starch than in soy extract-glucose medium. The optimum pH for growth in soy-glucose medium varied with the fungal species. With the lowering of pH, the yields of *Trametes versicolor* and *Phellinus igniarius* decreased gradually. The growth curve for *Stereum purpureum* was different; at pH 7.0 and 5.0 very poor growth occurred.

Several workers have studied the fermentation of wood-rotting fungi. We have reported on the cultivation and starch-decomposing activity of *Stereum purpureum* [9, 10]. JENNISON *et al.* studied the nutrition requirements [3, 4], growth [1], amino acid composition [2] and vitamin content [5], of submerged mycelium of basidiomycetes. BROCK [6], BLOCK [8] and WILLIAM *et al.* [7] examined the utilization of various carbon sources.

The present paper deals with studies on the influence of the initial pH on the homogeneous growth of *Stereum purpureum*, *Trametes versicolor* and *Phellinus igniarius* in glucose medium seeded with vegetative mycelium.

The purpose of the experiments was to determine the optimum pH for growth of cultures which had already lost their pellet producing capacity. In addition, an attempt has been made to find the best medium for fermentation.

Materials and methods

Media. Ingredients and preparation of media Sp—1, Cs—60 and Z/8 have been described previously [9].

Sterility testing was performed as described previously [9].

Inoculum. Agar pieces 2 cm^2 in size were cut from plates with surface cultures of the organisms and seeded into Z/8 medium. The cultures were shaken for 6 days at 30°C, then subcultured 5 times in the same medium at 3 day intervals. Cultures grown in this manner produced no pellets in glucose medium. The fermentation media were inoculated with 10 ml portions of such cultures.

Fermentation. Starch-soy medium Z/8 at pH 7.0 served as a control. Aliquots of soy-extract glucose medium Cs—60 were adjusted before sterilization to pH 7.0, 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0. Three parallel flasks of each pH value were seeded with 10 ml aliquots of inoculum and shaken for 3 days at 30°C, then the dry weight of mycelium present in the fermentation fluid was determined.

Dry weight determination. Pieces of filter paper were dried at 105°C for 2 hours and weighed on an analytical balance. Fifteen ml samples taken from each of the three parallel

cultures were pooled. Three 10 ml aliquots of the pooled sample were filtered through the weighed filter papers. After washing 3 times with distilled water, the filter papers were dried at 105°C for 2 hours, then weighed. The 3 parallel determinations were averaged and expressed as g/100 ml medium. The limit of error was ± 5 per cent.

Results

The results obtained in medium Cs-60 are presented in Fig. 1.

It is seen that in the examined pH range the growth of *Stereum purpureum* in soy extract glucose medium was very irregular. The best growth was obtained at pH 6.5, the poorest at pH 5.0.

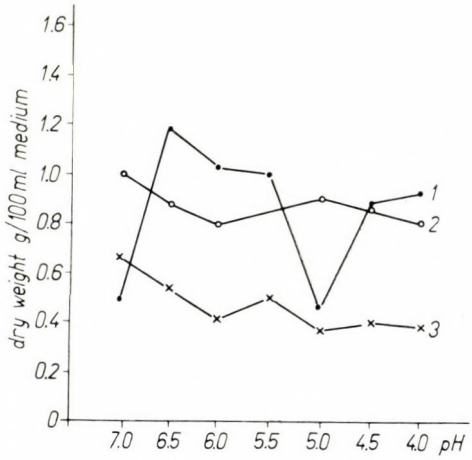

Fig. 1 (1) *Stereum purpureum* (2) *Trametes versicolor* (3) *Phellinus igniarius*

With *Trametes versicolor* the poorest growth was observed at pH 6.0 and 4.0.

With the lowering of the pH, the mycelium content of *Phellinus igniarius* cultures decreased gradually.

Glucose was utilized by all the examined organisms.

Table I shows the percentage of dry weight mycelium contents in medium Cs-60 as compared with the 100 per cent value obtained in control medium Z/8.

It is seen that *Stereum purpureum* grew poorly in glucose medium. The maximum mycelium yields at pH 6.5 were only 68 per cent of the control culture.

In glucose medium at pH 7.0 *Trametes versicolor* grew considerably better than *Stereum purpureum*. However, the maximum yields amounted to only 72 per cent of the control.

In medium Cs-60 the slowest growth was exerted by *Phellinus igniarius*. Highest yields (40 per cent) were obtained at pH 7.0.

It was striking that all the three species grew better in the control medium (Z/8) than in medium Cs-60.

Table I

Mycelium yield at various pH

Medium	pH	Percentage of dry weight mycelium contents		
		Stereum purpureum	*Trametes versicolor*	*Phellinus igniarius*
Z/8	7.0	100	100	100
Cs-60	7.0	28	72	40
	6.5	68	58	33
	6.0	58	53	25
	5.5	58	60	30
	5.0	26	63	23
	4.5	51	56	20
	4.0	53	53	23

Discussion

The examined three wood-rotting fungi, *Stereum purpureum*, *Trametes versicolor* and *Phellinus igniarius* grew uniformly well in medium Z/8 (Fig. 2). Accordingly, this medium is advantageous in that it ensures a shorter fermentation period. In addition, as it has already been shown [9], some subcultures in medium Z/8 are sufficient to preserve the dispersity of the culture.

The fact that the three species produced homogeneous cultures in medium Cs-60, was due to a previous stabilization of the inoculum by prolonged subcultivation in medium Z/8. When medium Cs-60 was seeded with direct surface cultures, at some pH values pellets were formed [9].

As to the optimal pH range of growth, measurable multiplication occurred between pH 7.0 and 4.0. The degree of growth, as expressed by the dry weight of the mycelium, depended on the initial pH value.

In medium Cs-60 *Trametes versicolor* and *Phellinus igniarius* grew best at pH 7.0. *Stereum purpureum* gave the poorest yields at pH 7.0 and 5.0. It would appear that the pH optimum of growth in a given medium varies with the fungal species.

Acknowledgement. The author is indebted to *Mrs.* G. SOMOGYI, *Miss* L. ROCKENBAUER and *Mrs.* L. PÁSZTOR for technical assistance.

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AMINO ACID SENSITIVITY OF STRAINS DERIVED FROM ESCHERICHIA COLI K_{12}

By

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Summary. A phenomenon similar to the amino acid inhibition observed by ALFÖLDI *et al.* in relaxed strains could be demonstrated with several stringent strains. These inhibitions were different from the feedback inhibition by valine. The lag-phase caused by the shift down could be prevented by valine and isoleucine. It is assumed that leucine causes a disturbance in the derepression of enzymes synthesizing the branched-chain amino acids.

The growth on minimal medium of certain *Escherichia coli* strains is inhibited by several amino acids. Thus, TATUM [1] has shown that the growth of *E. coli* K_{12} strains is inhibited by valine and this inhibition can be suspended by isoleucine. The cause of this inhibitory effect according to UMBARGER and BROWN [2] is that valine inhibits the α-acetolactic acid-forming enzyme which participates in the biosynthesis of valine and isoleucine; the presence of valine thus results in isoleucine deficiency. LEAVITT and UMBARGER [3] have shown that the enzyme present in valine-resistant mutants is not sensitive to valine, confirming by this the above supposition.

A new type of inhibition has been described by ALFÖLDI *et al.* [4]. In experiments with relaxed variants of *Escherichia coli* (BOREK *et al.* [5]) they found that if these bacteria were grown on complete medium for two hours then washed and plated on minimal medium, only 10 per cent of the bacteria formed colonies. This colony-forming ability is further decreased by a number of amino acids, first of all by branched-chain amino acids or hydroxy-amino acids participating in the biosynthesis of the former. ALFÖLDI and KEREKES [6] have demonstrated that these inhibitory effects can be overcome with combinations of members of this amino acid group but failed to observe a similar phenomenon in the case of stringent strains.

In the present paper the amino acid sensitivity of one relaxed and two stringent strains of *Escherichia coli* K_{12} was examined by a method different from that of the authors cited above. Since the strongest inhibitory effect was exerted by leucine, this amino acid was studied in detail.

Materials and methods

Strains. The following strains of *Escherichia coli* K_{12} were used: λ 28 RC^{str} (according to dr. R. HILL's determination) Hfr CAVALLI met^- RC^{rel}, 58—161 LEDERBERG met^- $biotin^-$ RC^{str}.

Experimental conditions. Bacteria grown for 16 hours on complete medium (broth) were inoculated into 10 volumes of the same medium. After two hours incubation the cultures were washed twice with distilled water and an inoculum of 10^3 cell/ml concentration was transferred into the minimal medium described by DAVIS and MINGIOLI [7] supplemented with a growth factor in the case of auxotrophic strains. After inoculation the growth-curve was determined by taking samples at intervals and plating them on complete medium. The experiments were carried out at 37°C. Any change in this standard method is indicated in the text.

The amino acids used were of L-configuration and of analytical purity.

Results

Demonstration of the inhibitory effect of leucine.

The effect of leucine upon the growth of the three mentioned strains has been examined. The results are shown in Fig. 1. It can be seen that leucine inhibited growth of all the three strains, and this inhibitory effect manifested itself in a lengthening of the lag-phase. The stringent K_{12} λ-28 strain was about 25 times more sensitive than the other two strains. After a similar lag-phase, the three strains grew at the same rate on minimal medium.

The effect of various amounts of leucine on strain K_{12} λ-28, the one most sensitive to leucine, was studied in detail (Fig. 2). It was observed that the lag-phase increased from 3 to 8 hours when 1 μg/ml leucine had been added to the minimal medium; 2.5 and 5 μg/ml leucine increased this period to 12 and 25 hours, respectively, and in the presence of 25 μg/ml leucine the viable

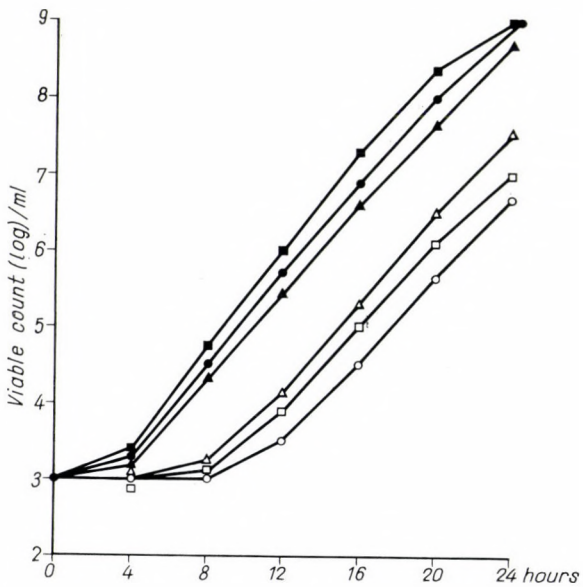

Fig. 1. Leucine sensitivity of various strains originating from *E. coli* K_{12}. Filled symbols: leucine-free medium. Empty symbols: leucin-containing medium. Circles: K_{12} λ-28, squares: K_{12} Hfr. CAVALLI, triangles: K_{12} 58—161 LEDENBERG. Leucine concentration 1 μg/ml with strain K_{12} λ-28, while 25 μg/ml in the other two cases

count did not increase during 36 hours. The above data represent the average values of numerous experiments; the small scatterings observed were probably due to the effectiveness of washing. In the case of the two strains less sensitive to leucine, the lag-phase was only slightly prolonged by higher amounts of leucine and complete inhibition could not be observed even at a concentration of 200 μg/ml.

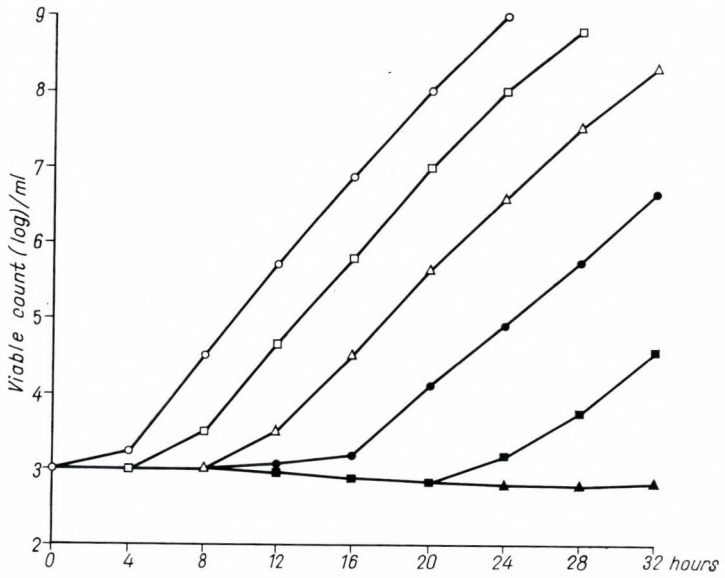

Fig. 2. Inhibition of growth of strain K_{12} λ-28 at various leucine concentrations. Media contained: ○——○ 0; □——□ 0.5; △——△ 1; ●——● 2.5; ■——■ 5; ▲——▲ 10 μg/ml of leucine

When in similar experiments the two first inoculations had been made on minimal medium, proliferation started without a lag-phase immediately after inoculation, and an inhibitory effect manifesting itself in the prolongation of proliferation could be detected only at considerably higher leucine concentrations (Fig. 3).

ALFÖLDI *et al.* [4] observed that the time of growth on complete medium had a pronounced effect on amino acid sensitivity. Examining the effect of the age of the culture, used as inoculum, we observed a similar phenomenon, *i.e.* leucine sensitivity diminished after incubation periods longer than two hours (Fig. 4).

The fact that in the presence of leucine, proliferation starts after a prolonged lag-phase, was thought to be due to the proliferation of leucine-resistant cells. Therefore, a culture grown in the presence of 2.5 μg/ml leucine with a lag-

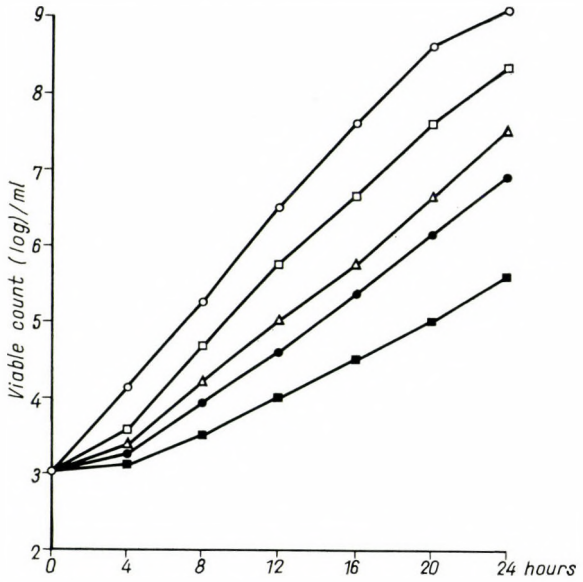

Fig. 3. Inhibition of growth of strain K_{12} λ-28 at various leucine concentrations in case of inocula grown on minimal medium. Media contained: ○——○ 0; □——□ 25; △——△ 50; ●——● 75; ■——■ 100 μg/ml of leucine

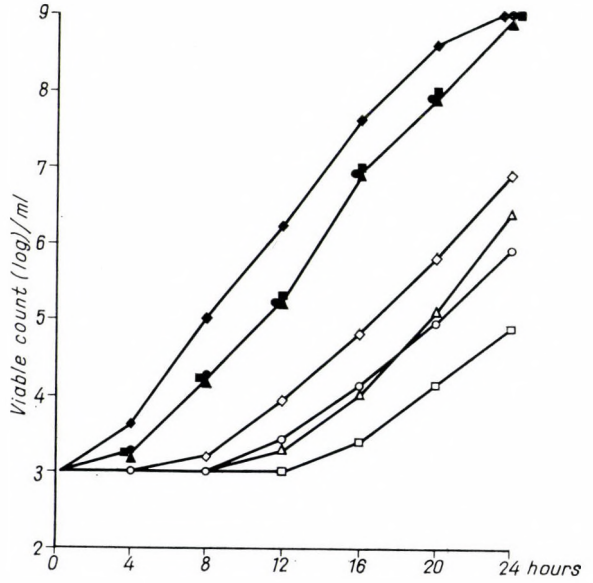

Fig. 4. Effect of growth period on leucine-sensitivity of strain K_{12} λ-28. Filled symbols: leucine-free medium. Open symbols: medium containing 2.5 μg/ml leucine. Inocula grown: circles, 0 hour; squares, 2 hours; triangles, 3 hours; rhomb, 5 hours; these inocula originated from a 16-hour-old culture in every case

phase of 12 hours was plated and the leucine-sensitivity of subcultures isolated from the colonies was determined. Leucine-sensitivity remained unchanged, thus the growth following the prolonged lag-phase cannot be attributed to selection.

Inhibitory effect of various amino acids on the growth of E. coli K 12 λ-28. The effect of natural amino acids on the growth of strain K_{12} λ-28 has been examined under conditions optimal for leucine inhibition. In addition to leu-

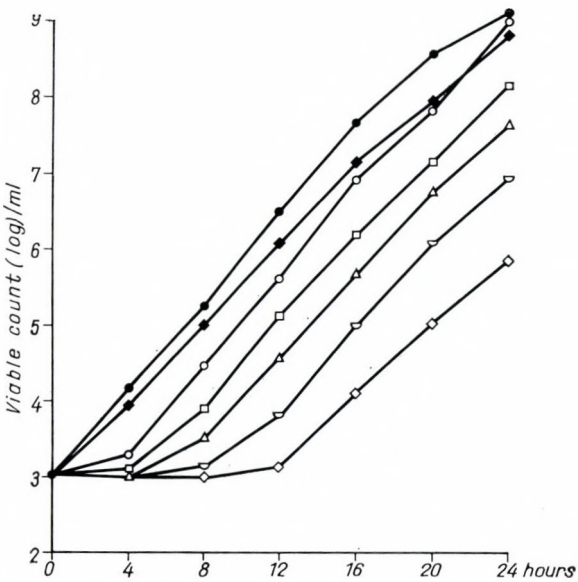

Fig. 5. Effect of isoleucine on growth of strain K_{12} λ-28. Filled symbols: inoculum grown on minimal medium. Empty symbols: inoculum grown on complete medium. Media contained: circles, 0; squares, 25; triangles, 50; half-circles, 100; rhomb, 200 μg/ml of isoleucine

cine, growth was inhibited also by L-valine, L-serine, L-threonine, and L-methionine. Results obtained with isoleucine and methionine are indicated in Figs. 5 and 6, respectively. Similarly to leucine both amino acids brought about the inhibition by prolonging the lag-phase, but at a concentration one order of magnitude higher than leucine. The extent of inhibition depended on the previous treatment of the inoculum. Small quantities (0.25 μg/ml) of leucine and isoleucine showed identical slight inhibitory effect.

The hydroxyamino acids (serine and threonine) inhibited growth by prolonging the lag-phase, similarly to leucine, isoleucine and methionine (Figs. 7, 8). Serine was more effective than threonine. At higher amino acid consentrations, a temporary decrease preceding the increase in viable count was observed. On the basis of the growth curve it could be assumed that a multiplication of resistant variants took place. Repeated tests revealed no

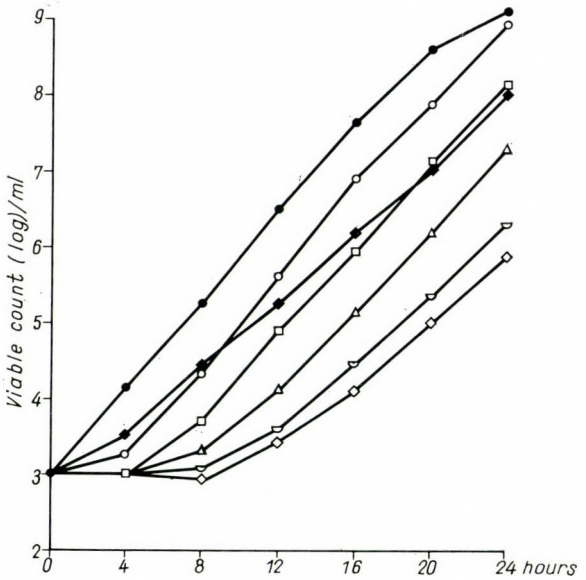

Fig. 6. Effect of methionine on growth of strain K_{12} λ-28. Filled symbols: inoculum grown on minimal medium. Empty symbols: inoculum grown on complete medium. Media contained: circles, 0; squares, 25; triangles, 50; half-circles, 100; rhomb, 200 μg/ml of methionine

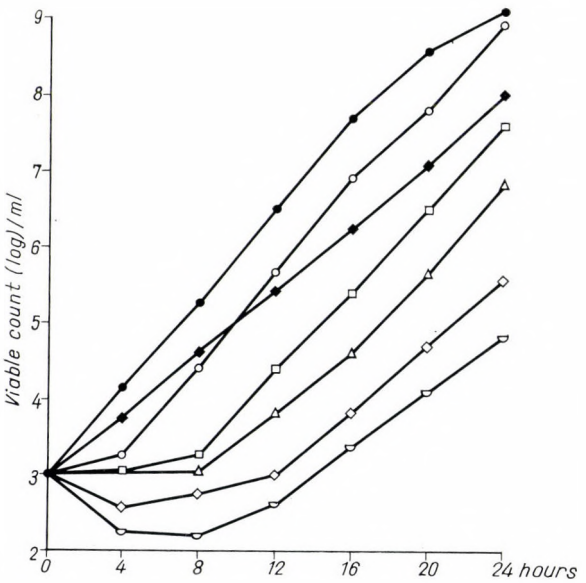

Fig. 7. Effect of serine on growth of K_{12} λ-28. Filled symbols: inoculum grown on minimal medium. Empty symbols: inoculum grown on complete medium. Media contained: circles, 0; squares, 5; triangles, 12.5; rhomb, 25; half-circles, 50 μg/ml of serine

increase in resistance. Our observation that the inhibition depends on the medium of the inoculum, relates also to the similarity of the inhibitory characteristics of hydroxyamino acids and leucine.

Valine showed an entirely different type of inhibition (Fig. 9). In this case the prolongation of the lag-phase was not so pronounced but the rate of proliferation decreased considerably or eventually ceased. The effect of valine could be observed also with cells grown on minimal medium.

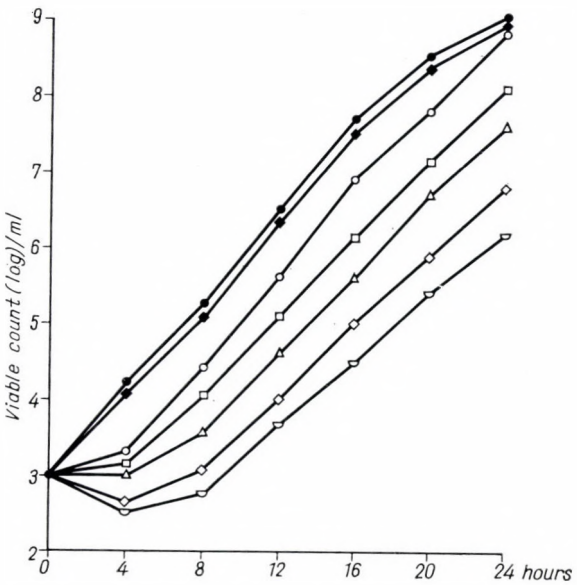

Fig. 8. Effect of threonine on growth of K_{12} λ-28. Filled symbols: inoculum grown on minimal medium. Empty symbols: inoculum grown on complete medium. Media contained: circles 0; squares, 5; triangles, 12.5; rhomb, 25; half-circles, 50 μg/ml of threonine

The effect of amino acids upon the growth of K_1 Hfr CAVALLI and K_{12} 58—161 LEDERBERG has been examined. Leucine and methionine caused no inhibition at a concentration of 200 μg/ml. Inhibitory effects were demonstrated with serine and threonine at concentrations above 50—100 μg/ml, but these strains were less sensitive than strains $K_{12}\lambda$-28. On the contrary, there was no significant difference in the valine-sensitivity of the three strains. The inhibitory concentration was between 0.1—0.5 μg/ml.

In the case of $K_{12}\lambda$-28, the other natural amino acids at a concentration of 200 μg/ml had no substantial effect on growth. Proliferation time was slightly decreased by tyrosine, aspartic acid, and glutamic acid, whereas tryptophan, histidine, proline, ornithine, citrulline, arginine and lysine were ineffective. Proliferation time was slightly increased by glycine, alanine, cysteine and

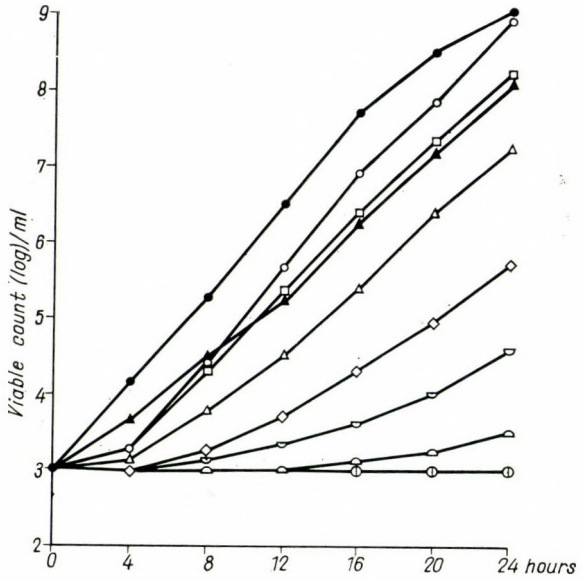

Fig. 9. Effect of valine on growth of K_{12} λ-28. Filled symbols: inoculum grown on minimal medium. Empty symbols: inoculum grown on complete medium. Media contained: circles, 0; squares, 0.005; triangles 0.0075; rhomb, 0.01; ⌓——⌓, 0.025; ⌒——⌒, 0.05; ⌽——⌽, 01. μg/ml of valine

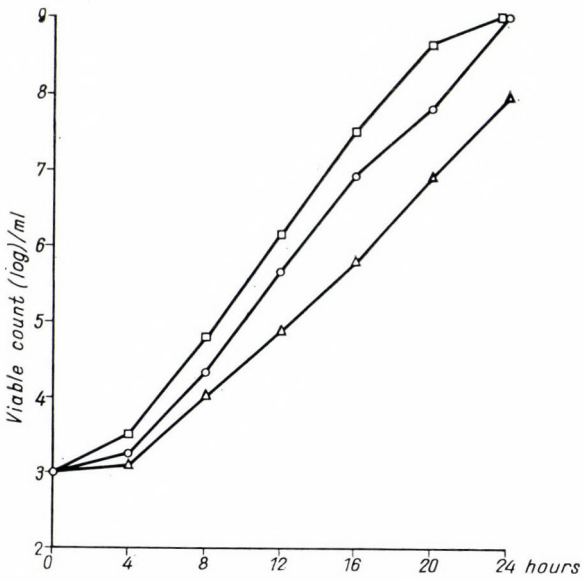

Fig. 10. Effect of tyrosine, glycine and proline on growth of strain K_{12} λ-28. ○——○: control or proline; □——□: tyrosine; △——△: glycine. The medium contained 200 μg/ml of the amino acids

phenylalanine. The effect of one member of each of the three groups is shown in Fig. 10.

The effect of combinations of branched-chain amino acids on the growth of E. coli strain K_{12} λ-28. First the effect on growth paired combinations of the three branched-chain amino acids was studied. Leucine and isoleucine mutually decreased each other's inhibitory effect (Fig. 11). The effect of 25 μg/ml leucine or isoleucine was considerably reduced by 1 μg/ml of isoleucine

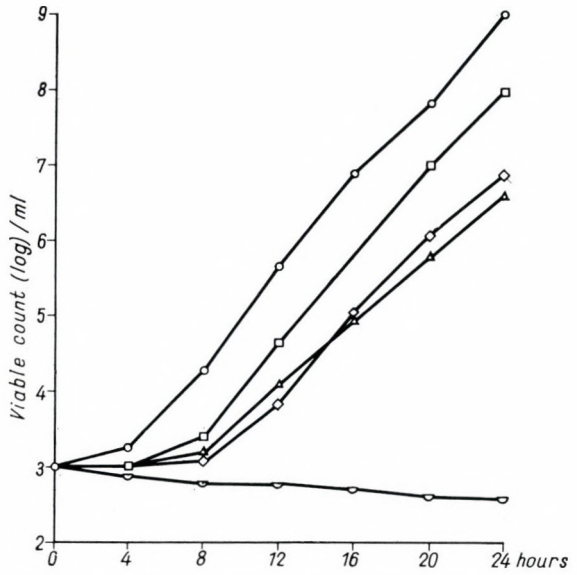

Fig. 11. Effect of isoleucine and leucine combinations on growth of strain K_{12} λ-28. ○——○: control; □——□: 25 μg/ml isoleucine and 1—25 μg/ml leucine; △——△: 25 μg/ml leucine and 1 μg/ml isoleucine; ◇——◇: 25 μg/ml isoleucine; ⌒——⌒: 25 μg/ml leucine

and leucine, respectively. With this combination the lag-phase obtained on amino acid-free minimal medium could, however, not be attained. The mutual protecting effect depended on the relative concentrations, thus for partial prevention of the effect of 200 μg/ml leucine a concentration of above 10 μg/ml of isoleucine was needed, whereas in the presence of 2.5 μg/ml of leucine 0.1 μg/ml of isoleucine was still effective.

Entirely different effects were obtained with the combination of valine and isoleucine (Fig. 12). The protecting effect was again mutual. The effect of 25 μg/ml of valine was prevented by as little as 1 μg/ml of isoleucine. In this case growth started without a lag-phase and a shift down effect; when the level of 10^8 cell/ml had been attained the increase in viable count stopped. On increasing the concentration of isoleucine the maximum viable count rose, but the lag-phase became more and more pronounced, though even in the

presence of 25 μg/ml leucine it did not or did only slightly exceed that of the control culture. If 1 μg/ml valine was added to 25 μg/ml isoleucine there appeared a lag-phase somewhat longer than the control but considerably shorter than that observed on a medium containing isoleucine only.

If valine and isoleucine were applied together at the same concentration, they had a stimulating effect on growth rate.

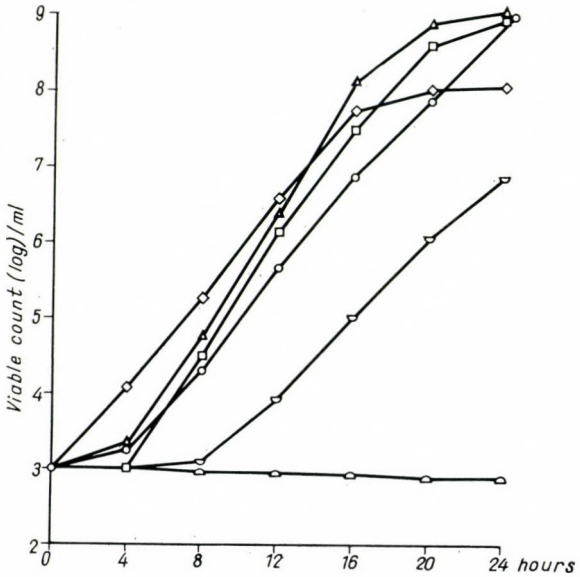

Fig. 12. Effect of isoleucine and valine combinations on growth of strain K_{12} λ-28. ○——○: control; □——□: 25 μg/ml isoleucine and 1 μg/ml valine; △——△: 25 μg/ml isoleucine and 25 μg/ml valine; ◇——◇: 1 μg/ml isoleucine and 25 μg/ml valine; ⌓——⌓: 25 μg/ml isoleucine; ⌒——⌒: 25 μg/ml valine

While an antagonistic effect could be demonstrated between valine and isoleucine and between leucine and isoleucine, with the combination of valine and leucine such an effect could not be detected; moreover, when valine and leucine were applied in concentrations producing only partial inhibition, a slight synergetic effect could be observed in some experiments.

In the experiment shown in Fig. 13 the simultaneous effect of the three amino acids was examined. When leucine was added to 25—25 μg/ml of isoleucine and valine, the lag-phase due to the shifting down was abolished by 1 μg/ml leucine; the culture was growing as if it had been inoculated with cells grown on minimal medium. In the case of 25—25 μg/ml isoleucine + leucine the addition of 1 μg/ml valine diminished the lag-phase to a value corresponding to the control. In the presence of 25—25 μg/ml leucine + valine, the addition of 1 μg/ml isoleucine exerted less of a protection than if it had been given to 25 μg/ml leucine or to the same amount of valine alone.

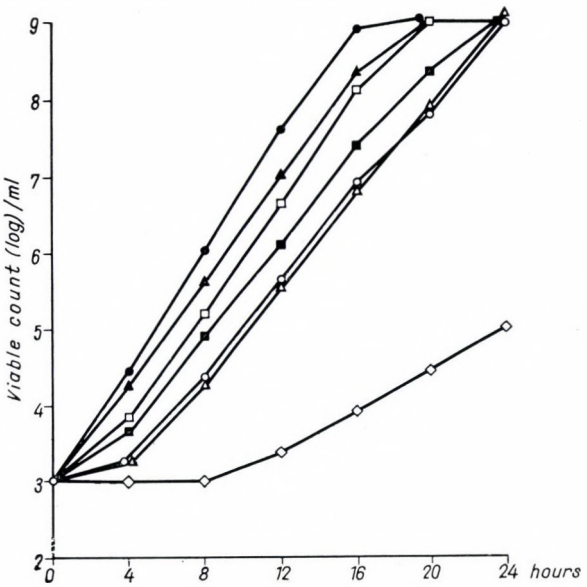

Fig. 13. Simultaneous effect of isoleucine, valine and leucine on growth of strain K_{12} λ-28. ○——○: control; □——□: 25 μg/ml isoleucine, 25 μg/ml valine and 1 μg/ml leucine; △——△: 25 μg/ml isoleucine, 25 μg/ml leucine and 1 μg/ml valine; ◇——◇: 25 μg/ml valine, 25 μg/ml leucine and 1 μg/ml isoleucine. The three amino acids were added in equal amounts: at concentrations of ●——● 200; ▲——▲ 25; ■——■ 2—5 μg/ml

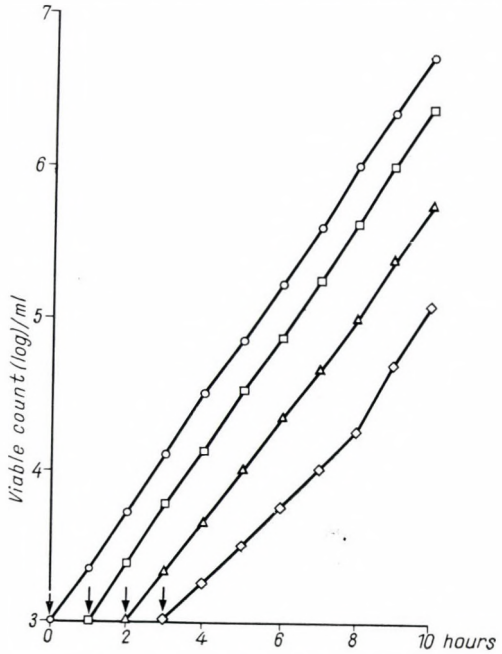

Fig. 14. Protecting effect against leucine of isoleucine and valine added with delay. To every culture 200 μg/ml of leucine was added at 0 time. Addition of isoleucine and valine (200 μg/ml of each every time) with a delay of ○——○ 0; □——□ 1; △——△ 2; ◇——◇ 3, hours

The behaviour of leucine- and valine-resistant mutants. When *E. coli* strain K_{12} λ-28 was plated on solid minimal medium containing 200 μg/ml leucine or 10 μg/ml valine, and after two-day incubation the resistant colonies were isolated, it could be observed that all of the valine-resistant colonies were more resistant to leucine than the original strain. The resistant variants obtained from the leucine-containing plates could be divided into two groups: one of them has lost its sensitivity only to leucine (200 μg/ml) while it showed resistance not only to the same amount of leucine but also to 1 to 50 μg/ml of valine. These results are in agreement with the recent experiments of ALFÖLDI [8].

Discussion

The inhibitory effects exerted by leucine and other amino acids show some relationship with the phenomenon described by ALFÖLDI *et al.* [4]. These authors found, however, only relaxed recombinants to be leucine-sensitive. In our experiments significant differences in leucine-sensitivity have been found between a stringent and a closely related relaxed strain, and it was a stringent strain that proved to be most sensitive to leucine. This suggests that amino acid sensitivity does not by all means depend on the presence of a certain allel of the RC locus.

According to the catholic inducer theory of ALFÖLDI and STENT [4], the amino acid-free sRNA as the repressor of RNA synthesis, is activated by a non-cognate amino acid or by some other substance of related structure, and leucine exerts its effect in a way analogous to that of this catholic inducer. As to the cause of the differences between stringent and relaxed strains, in their opinion the following alternatives seem to be possible: in the stringent strain the repressor is not sensitive to the catholic inducer; the enzyme transferring the catholic inducer is different; or, else, there is no catholic inducer present. The leucine-sensitivity of the stringent strains rules out the first two possibilities: the stringent and relaxed strains may differ only in the production of the catholic inducer, but not in repressor-sensitivity or in the enzyme carrying out the reaction.

Recently ALFÖLDI and KEREKES [6] completing their previous theory, have assumed that leucine increases synthesis of the catholic inducer through a false feed-back inhibition and thus more relaxing RNA can be accumulated in a relaxed strain. On the basis of this hypothesis, our present observation means that synthesis of catholic inducer may take place in a stringent strain in the presence of leucine or some other amino acid similar in effect.

A strong leucine-type inhibition could be elicited when, in the repressed state, the cells were transferred from complete medium to minimal medium. Another prerequisite of the inhibition is that the amino acid should be given

to the culture at the beginning of the lag-phase. Two hours later, at a time when growth has not yet started (at about 2/3 of the lag-phase), leucine-sensitivity is so reduced as if the inoculum had grown on minimal medium. The inhibitory effects were obtained with branched-chain amino acids or with amino acids participating in their biosynthesis directly or indirectly (methionine). It has been shown (UMBARGER and BROWN [2], HALPERN and UMBARGER [9], HORVÁTH *et al.* [10]) on the other hand, that when various bacterial strains are inoculated into synthetic medium, the amount of enzymes participating in the biosynthesis of the branched-chain amino acids significantly increases. These two phenomena might be brought into connection. This assumption is supported by the observation that, even if inoculated from complete medium, growth starts without a lag-phase in the presence of the branched-chain amino acids, or even valine and isoleucine, since leucine only prevents toxic effect of the isoleucine concentration required for attaining the maximum population. All these suggest that the derepression of enzymes participating in the synthesis of valine and isoleucine is the limiting process which gives rise to the shift down lag-phase and which is disturbed by the leucine-type inhibitions indirectly (thus not by direct repression or feed-back).

The indirect character of leucine-inhibition is supported by the differences which appear on comparison with the feed-back type valine-inhibition. While with respect to leucine and isoleucine there was a marked difference between strain K_{12} λ-28 and the strains Hfr CAVALLI and 58—161 LEDERBERG, in valine-sensitivity such a difference could not be established. Valine-sensitivity did not depend on the medium of the inoculum; the effect of valine, in contrast to that of leucine, manifested itself first of all in a reduction of growth rate. Finally the leucine-resistant variants differ in valine-resistance. While all these circumstances point to the different mechanism of leucine-inhibition, the leucine-resistance of valine-resistant variants suggests that valine may also have an inhibitory effect, which, however, is masked by the feed-back effect active at low concentrations already.

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TRANSDUCTION IN BACILLUS SUBTILIS

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Summary. (i) Comparative examinations have shown that the temperate subtilis phages can be divided into two groups. *B. subtilis* strain NRS 231 was an adequate common host sensitive to all the examined temperate phages. Owing to certain technical advantages this strain was found suitable for the titration of temperate subtilis phages.

(ii) Some cultural differences have been revealed among substrains of *B. subtilis* 168 ind$^-$ auxotrophs maintained in various laboratories. Cells in one of these cultures were partly capable and partly incapable of using ammonia. Ammonia assimilation was transducible to ammonia negative bacteria.

(iii) After mitomycin C or ultraviolet ray induction the examined *B. subtilis* strains liberated "bacteriocin"-like principles. This finding is probably analogous to that of Seaman *et al.* concerning PBSX defective phages. On the basis of bacteriocin production the examined strains were divided into two groups.

(iv) Several temperate phages isolated in our laboratory were presumably identical with phage PBS 1. Phage SP 10 differed from these agents both in antigenic structure and in host range.

(v) Transduction by some lysates of PBS 1-type phages was observed at frequencies of the order of 10^{-6}. Considerably more and less effective phage materials were yielded by some lysogenic transductants. Thus transducing phages active in the order of 10^{-4} were prepared.

(vi) Transduction frequency, in addition to the properties of the phage, was influenced by the physiological condition of bacteria.

(vii) Transduction of indole and histidine loci was studied by use of his$^-$ derivatives of strain 168 ind$^-$.

We have recently shown [1] that by means of the streptomycin method [2] subtilis phages can be isolated from the majority of garden soil samples. Among these phages the temperate ones were able to transduce various genetic markers [3]. In the isolation and assay of these phages some difficulties were encountered, because the usual complete media (yeast extract — peptone agar) overlayered with soft agar, frequently yielded undefinite plaques. Similar difficulties were described by Takahashi [4] and Thorne [5]. These temperate subtilis phages could, however, be assayed adequately if instead of the complete medium a synthetic, glutamic acid and glycerol-containing nutrient agar and *B. subtilis* strain Marburg (Yale) were used [3].

The mentioned difficulties indicate that the methods applied for studying temperate-type subtilis phages need some revision. The purpose of the present work was on the one hand to elucidate these technical problems, and, on the other, to compare the behaviour of phages and *B. subtilis* strains isolated and maintained in various laboratories.

Materials and methods

Bacterial strains. Strain *Bacillus subtilis* Marburg, Yale was received from Dr. SCHAEFFER's collection, Paris. The strain is probably identical with the prototrophic strain of BURKHOLDER and GILES [6].

Auxotrophic strains 168 ind$^-$ maintained in various laboratories were used in comparative examinations. Although all of these cultures were received bearing the same designation and were probably derivatives of the same organism, their detailed examination revealed some differences. Therefore the laboratory from which the corresponding auxotroph was received, will be noted. The strains used in this study are listed in Table I.

Phages. In addition to 5 turbid plaque-producing and transducing phages (1 KT, 3 NT, 4 P, 6a and 6b) described previously, similar, recently isolated phages were also studied. These phages, 7 T, 8 Tf, 9 TR, 10a and 10b, produced also turbid plaques and were able to transduce the indole marker. Phages 3 NV, 4L, 10 L and 10 W, which produced clear plaques, did not transduce this marker.

Phage SP 10 was kindly supplied by Dr. C. B. THORNE. Phage PBS 1 isolated and studied by TAKAHASHI [7, 4], was obtained from Dr. P. SCHAEFFER's collection.

Reagents. Analytical grade commercial indole, DL-tryptophan and histidine were used. Commercial L-glutamic acid was recrystallized twice before use.

Media. For maintenance of strains and other experimental purposes YP (yeast extract — peptone) medium was used. Routine preparation of the medium is at present carried out as follows:

Baker's yeast weighing 40 kg is suspended in 40 litres of tap water, then the volume is made up to 100 litres. After autoclaving at 120°C for 30 minutes and cooling, the suspension is centrifuged and the supernatant filtered through asbestos filter. The extract is distributed at 2 litre amounts and sterilized by autoclaving at 115°C for 30 minutes. YP medium contains 100 ml of yeast extract, 10 g of peptone (Richter, Budapest), and 5 g of NaCl in 1 litre. The pH is adjusted to 7.4, then the medium is filtered, distributed and autoclaved.

MM (minimal medium): NH_4Cl, 2 g; K_2HPO_4, 1.4 g; KH_2PO_4, 0.6 g; sodium citrate, 1 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $MnSO_4 \cdot 4H_2O$, 0.05 g; distilled water, 1000 ml; pH 7.2; glucose, 0.5 per cent.

GGM (glutamic acid-glycerol medium) was prepared as described previously [3].

gGM (glutamic acid-glycerol medium) differed from GGM only in containing 0.2 g of glutamic acid per litre.

Synthetic media were always prepared in the double strength form. Before use fluid media were supplemented with equal amounts of distilled water, solid media with equal amounts of 3 per cent aqueous agar. Glucose was sterilized separately and incorporated in MM medium to give final concentrations of 0.5 per cent. Glutamic acid-glycerol media contained no glucose.

Routine preparation of phage materials. Fluid YP medium distributed at 15 ml amounts in 100 ml Erlenmeyer flasks was seeded from one turbid plaque. Thus the inoculum contained free phage particles and lysogenic bacteria. Unless indicated otherwise, the plaque was subcultured from Yale indicator plate. Phage SP 10 was transferred into liquid medium from plaques obtained on prototrophic W_{23} cells. The inoculated media were gently shaken through a distance of 9.5 cm at a rate of 75 cycles per minute for 8—10 hours in a 37°C water bath. After incubation the cultures were left to stand at room temperature overnight, then centrifuged and passed through glass filters as described in a previous paper [3].

Phage preparations producing clear plaques were obtained in the usual manner on the Yale strain.

Titration of phage preparations. In previous experiments the wild-type Yale strain was used for estimating the number of plaque forming units. With GGM agar, tolerably good but not always precisely reproducible results were obtained. As this method involved the drying of assay plates, sometimes in consequence of an uneven distribution of moisture, in some parts of the plate the plaques were not sufficiently distinct. As for this the indicator strain was thought to be responsible, organisms more suitable than the Yale strain were sought for. Of several standard laboratory cultures strain NRS 231 was found adequate; this strain gave well demarcated plaques besides being more sensitive than the Yale strain. A further advantage was that NRS 231, unlike the Yale strain, was sensitive to phage SP 10.

To 0.1 ml phage dilution 0.9 ml exponential growth phase culture of strain NRS 231 was added. This suspension was obtained by preparing an 1 : 10 dilution of YP culture giving an optical density reading of 0.5. Phages were diluted with saline containing 20 per cent liquid YP medium. After the addition of 1 ml 65°C GGM medium containing 1 per cent agar, the phage-bacterium mixture was immediately poured over a GGM plate 10 cm in diameter. When

the layer had hardened, the lids were removed and the plates were dried upside down at 37°C for 20 minutes. Readings were performed after incubation at 37° C overnight.

Transduction experiments were carried out essentially in the same manner as described previously [3]. To 1 ml exponential growth phase YP culture 1 ml of phage preparation was added and the mixture was left to stand at room temperature for 20 minutes. Dilution of high titre phage preparations was carried out with liquid YP medium. Sometimes, because of the required high m.o.i. value, the amount of phage preparation had to be increased to 2—3 ml. The bacterium-phage mixture was centrifuged at 2000 r.p.m. The supernatant was discarded and the deposit was washed in 5 ml MM solution. After resuspending the bacteria in 1 ml MM solution, 0.1 ml diluted or undiluted samples were plated on suitable minimal medium. Readings were made after 40 to 48 hours incubation at 37° C.

Results

Cultural properties of indole auxotrophs.

Under certain cultural conditions the behaviour of 168 ind$^-$ strains, which had probably originated from one organism but were maintained in various laboratories, was not uniform. In colonial morphology all cultures were identical on YP agar. When grown on synthetic media as gGM or MM agar, different colonial forms appeared. The difference was not distinct on GGM agar containing 0.3 per cent glutamic acid, but it was striking on gGM agar which contained 0.02 per cent of this substance. Cultures grown on gGM agar containing 25 μg/ml indole were characterized as follows.

Strain S 168 *ind*$^-$. After 48 hours at 37°C two colonial types occurring in approximately equal numbers were noted: one type was characterized by rusty coloured colonies with entire edge, 3—4 mm in diameter, the other by smaller, flat, non-pigmented colonies with irregular edge. The two colonial variants differed in the utilization of ammonium salts: in contrast to that of the pigmented type, the nitrogen requirement of the non-pigmented form could not be supplied with these compounds. The latter form produced no colonies on MM agar and multiplied only on glutamic acid-containing medium. Both types grew, of course, abundantly on complete YP agar. Therefore the stock S 168 ind$^-$ strain contained ammonium-assimilating (Am$^+$) and ammonium-non-assimilating (Am$^-$) mutants. In case of the latter, aspartic acid or asparagine could be substituted for glutamic acid. With other amino acids such as histidine, glycine, alanine and threonine, no growth was observed.

The gGM subculture of pigment-producing Am$^+$ mutants was homogeneous even after prolonged incubation. The 48 hour Am$^-$ subculture consisted of small, seemingly homogeneous non-pigmented colonies. After 4 to 5 days pigment-producing secondary papillae had appeared which became larger on further incubation and caused the deformation of the colony. These papillae consisted of Am$^+$ mutants.

It would appear that the original S 168 ind$^-$ strain contained ammonium-utilizing cells and segregation of Am$^-$ mutants occurred during maintenance on complete media. However, as shown in further experiments, Am$^+$ $\rightarrow$ Am$^-$ mutation was reversible.

Table I

B. subtilis strains and their sources

Our designation	Source of culture
Marburg, Yale (prototrophic)+	Dr. P. SCHAEFFER (Paris)
S 168 ind−	,,
S 168 ind− ad− [21]	,,
L 168 ind−	Dr. J. LEDERBERG (Palo Alto)
T 168 ind−	Dr. C. B. THORNE (Oregon)
$W_{23}Sr$ (prototrophic)++	,,
NRS 231 (prototrophic)	Dr. R. GORDON (New Brunswick)
13 ind− his−	Dr. S. ZAMENHOF (New York)
26 ind− his−	,,
30 ind− his−	,,
31 ind− his−	,,
SB ind− his−	,,
SBI ind− his−	,,

+ In the text this prototroph is referred to as "Yale".
++ In the text this prototroph is referred to as "W_{23}".

Strain L 168 ind− produced on gGM medium small, convex, glistening, non-pigmented colonies, 1.5—2 mm in diameter. This strain utilized ammonium salts.

Strain T168 ind−. Unlike the former organism, strain T168 ind− produced definitely R-type colonies. The poorly pigmented culture was well distinguishable from the larger, darkly pigmented colonies of strain S168 ind− Am+. Cells of strain T168 ind− assimilated ammonium salts.

It should be noted that gGM agar colonies of the ammonium-utilizing Yale strain were perfectly similar to those of strain S 168 ind− Am+.

Accordingly, some differences were revealed among the mentioned indole auxotrophs, which had probably originated from one strain but were maintained in various laboratories.

Bacteriocin production by B. subtilis.

According to the personal communication of Dr. SCHAEFFER (Paris), ultraviolet irradiated *B. subtilis* strains produced bacteriocin-like agents. Electronmicroscopic studies revealed defective phages in the lysates of these strains. In lysates of mitomycin-induced *B. subtilis* 168 wild-type revertant cultures, SEAMAN *et al.* [8] showed the presence of genetically defective phages. These defective phages produced no plaques and only their bactericidal effect was pointing to their presence. In behaviour

defective phages therefore resemble bacteriocins. SEAMAN *et al.* used the "cured" derivative of the original strain as sensitive indicator culture. In accordance with these findings, in our experiments ultraviolet irradiation and more intensively, mitomycin treatment, caused the production of bacterio-

Table II

Activity spectrum of bacteriocins produced by some B. subtilis strains

Source of bacteriocin	Sensitivity of indicator strain		
	Yale	W_{23}	NRS 231
Yale	0	1 : 64	1 : 32
W_{23}	1 : 16	0	0
NRS 231	1 : 32	0	0

Fractions mean the highest effective dilution of bacteriocins; 0 = ineffective. Different derivatives of strain 168 behaved like the Yale strain. To 10 ml culture (O. D. = 0.25) 3 μg/ml mitomycin was added. After 30 minutes incubation the culture was washed by centrifugation and resuspended in liquid YP medium. The lysate obtained after a residual growth was used.

cin-like principles in all strains 168 ind$^-$ as well as in strains W_{23}, Yale and NRS 231. The bacteriocins were examined only for activity spectrum. Strains 168 and Yale produced seemingly identical bacteriocins, which differed from those of strains W_{23} and NRS 231 (Table II).

If it is assumed that the bacteriocins examined in this study are genetically defective phages, our strains produced various types of such agents. Strains W_{23} and NRS 231 seemed to elaborate similar defective prophages. Strains Yale and 168 carried identical defective prophages different from those liberated from the former strains. Neither of the lysates contained infective phage particles.

Comparative examination of subtilis phages.

The 10 turbid plaque-forming transducing phages isolated in our laboratory behaved identically and showed a complete resemblance to phage PBS 1. On indicator strain NRS 231 they formed morphologically similar turbid plaques measuring 4—5 mm in diameter and displaying well-defined edges. After 2 days in the centre of the plaque a secondary growth commenced and developed later into a flat central colony. At these phages produced uniform plaques on strains NRS 231, Yale, W_{23} as well as on the examined cultures of indole auxotroph 168. With all indicators identical e. o. p. values were obtained.

On the other hand, phage SP 10 produced no plaques on strains Yale and 168 ind$^-$. Identical e. o. p. values were found with strains W_{23} and NRS 231.

Differences in the antigenic structure of phages corresponded to host-range differences. The K values of anti-phage 6a immune serum were similar for homo-

logous and other phages: 6a = 630; 3 NT = 648; PBS 1 = 692. With the exception of phage SP 10, all transducing phages were presumably identical in antigenic structure. Anti-phage 6a serum did not neutralize phage SP 10, within the limits of the test's error, the K value was 0 in several experiments.

Clear plaque-forming subtilis phages 3 L, 3 NV, 10 L and 10 W were similarly not neutralized by anti-phage serum 6a. With anti-phage serum 4 L these phages reacted uniformly. The results are summarized in Table III.

Accordingly, the examined subtilis phages could definitely be distinguished on the basis of the mentioned properties. In view of the antigenic difference observed, phages producing clear plaques could not be regarded as virulent mutants of temperate phages. The host range of these phages also indicated that they comprised a separate type of subtilis phages. Phage PBS 1 originating from a Canadian soil sample, resembled in every respect the phages isolated from local garden soil in our laboratory.

Table III

Classification of 16 different subtilis phages according to various properties

Phage	Plaque morphology	E. o. p.				Neutralization		Transduction of indole marker
		Yale	S 168 ind⁻	W_{23}	NRS 231	antiphage serum		
						6a	4L	
1 Kt; 3 NT; 4 P; 6a; 6b; 7T; 8tf; 9TR; 10a; 10b	Turbid	1.0	1.0	1.0	1.0	+	—	+
PBS 1	Turbid	1.0	1.0	1.0	1.0	+	—	+
SP 10	Turbid	0	0	1.0	1.0	—	—	+
4L; 3NV; 10L; 10W	Clear	1.0	1.0	0.1—0.001	0.1—0.001	—	+	—

0 = no reaction with undiluted lysate

These phages, however, differed from SP 10 in host range as well as in antigenic structure.

Frequency of transduction of the indole marker.

Behaviour of various phages. By temperate phages isolated in our laboratory the indole marker was transduced at frequencies ranging from 10^{-6} to 10^{-7}/PFU. Throughout these experiments the prototrophic Yale strain was used as donor. It should be noted that in Table IV the results obtained with lysates stored for not more than one week were only included. Phages 3 NT and PBS 1 propagated on strain Yale showed transducing frequencies in the order of 10^{-6}/PFU. A somewhat similar transducing frequency was exerted

by SP 10 propagated on strain W_{23}. The results of transduction experiments with 14 different lysates are summarized in Table IV.

In addition to the above phage preparations, other 3 NT lysates were also examined. The results of these experiments were omitted from Table IV, as these phages were stored for more than a few weeks. As compared with the others, one of these phage materials produced an one exponent higher transduction frequency. Subcultures of this phage yielded preparations displaying the usual frequency. By changing the medium, incubation temperature and aeration, several experiments were performed to obtain more active preparations. The transducing activity of the phages, however, remained invariably unaltered.

The frequency of transduction expressed in plaque-forming units was, of course, influenced by the multiplicity of infection. It is seen that at values 2 or less the frequency differences were within the limits of the test's error. As compared with results obtained at m. o. i. = 2, at m. o. i. = 10 the absolute number of transductants was also less. This was probably due to a lysis of a certain proportion of infected cells (Table V).

Table IV

Transduction frequencies obtained with different phage preparations

Phage	Protocol no.	Phage titre PFU/ml	m. o. i.	No. of transductants/PFU
3 NT	2/113	4×10^9	1.0	1.4×10^{-6}
	2/139	6×10^8	0.9	1.3×10^{-7}
	3/8	2×10^8	0.9	4.6×10^{-6}
	3/53	10^9	0.9	3.4×10^{-6}
	3/57	5×10^8	0.8	1.7×10^{-6}
	3/70	5×10^8	1.8	5.8×10^{-6}
	3/63	5×10^8	0.8	2.1×10^{-6}
PBS 1	1/61	3×10^8	1.5	8.3×10^{-6}
	5/17	6×10^8	1.0	1.7×10^{-6}
SP 10	3/53	5×10^8	0.5	1.6×10^{-6}
	3/56	3×10^8	0.3	2.2×10^{-6}
	3/6	2×10^8	0.5	2.1×10^{-6}
	3/37	10^8	0.1	4.8×10^{-6}
	2/142	10^9	2.0	8×10^{-8}

The number of transductants was examined by seeding on gGM agar

Association between the transduction frequency of indole marker and the physiological condition of bacteria. When S 168 ind$^-$ cells treated with phage 3 NT were plated on minimal media MM and gGM, the number of transductants was usually different. Time and temperature of adsorption were not responsible for this finding. The results varied with the age of the culture: young cultures

Table V

Frequency of transduction of strain S 168 ind$^-$ by phage 3 NT, at varying m. o. i. values

m. o. i.	Transductants/ml	No. of transductants/PFU
10	4.2×10^2	10^{-7}
2	1.2×10^3	1.5×10^{-6}
0.4	4.8×10^2	3×10^{-6}
0.008	1.4×10^2	4.3×10^{-6}
0.016	2×10^1	3×10^{-6}

Titre of phage preparation, 4×10^9/ml; bacterial count, 4×10^8/ml. Transductants were isolated on gGM plates.

gave generally more transductants on MM medium than on glutamic acid-containing gGM medium. The results were reproducible. The m. o. i. value also influenced the results. One such experiment is presented in Table VI.

The number of transductants was highly influenced by the age of the culture. In young cultures larger numbers of transductants appeared on MM than on gGM agar. Bacteria from exponential or stationary phase cultures behaved in an opposite manner. The cause of this phenomenon could not be elucidated.

Phage material with increased frequency transduction (IFT).

With phages prepared by the method described only some hundreds or at most one thousand transductants were obtained from 10^8 to 5×10^8 recipient bacteria. One 3 NT preparation yielded considerably more transductants.This observation indicated that phage populations more active than the average might be prepared and we succeeded in obtaining populations with increased activity from lysogenic clones by use of phages 3 NT and PBS 1 as follows.

A suspension of S 168 ind$^-$ cells was infected with phage 3 NT (m. o. i. = = 1), then plated on gGM medium. After 1 week, when the transductants were mainly in the sporulated form, the colonies were separately suspended and heated at 70° C for 5 minutes. The lysogenic spore preparation was then transferred into liquid YP medium and shaken at 37° C overnight. The transducing activity of phage materials obtained from various lysogenic clones was com-

Table VI

Transduction of the indole marker by phage 3 NT to strain S 168 ind⁻ at different stages of cultivation

Culture		m. o. i.	Number of transductants	
O. D.	Colony forming units/ml		gGM agar	MM agar
0.4	4.5×10^7	15.0	230	480
		1.5	160	430
		0.15	40	50
0.6	1×10^8	5.8	610	810
		1.9	640	820
		0.2	160	130
0.9	3.7×10^8	1.4	430	280
		0.9	620	550+
		0.1	180	200
1.1	5×10^8	1.4	950	280
		0.14	200	100

An exponential growth phase culture of strain S 168 ind⁻ in YP medium was sampled and examined for optical density and viable counts at different intervals. To aliquots of the samples, equal amounts of 3 NT lysate were added. After 30 minutes the suspensions were centrifuged, washed with MM medium and plated at 0.1 ml amounts on gGM and MM agar. + In contrast to this high value, in further experiments with older cultures less transductants were observed on MM medium than on glutamic acid-containing gGM medium.

pared. Table VII reveals the result of one of these experiments carried out with phage preparation obtained with 10 individual transductants.

The transduction frequency of the indole marker varied considerably with the different phage preparations. It should be mentioned that for the comparative examinations the recipient cells were obtained from one culture of strain S 168 ind⁻. There was no relation between the number of transductants and the phage titre of lysates. Accordingly, phage populations originating from different transductants were not uniform. Some lysogenic clones liberated more phage particles containing bacterial chromosomal material. The observed phenomenon cannot be compared with "specific transduction" since (1) when propagated in the usual manner, IFT preparations yielded phages with an average and not with an increased transducing activity; (2) IFT preparations transduced not only indole but also other markers at high frequencies.

The different activity of phage preparations is, therefore, not due to a genetic difference in phage particles, but to the character of the lysogenic

Table VII

Behaviour of phage preparations obtained from 10 different lysogenic transductants

Phage preparation	Titre PFU/ml	No. of transductants/ml bacterium	No. of transductants/PFU
1	5×10^9	2.5×10^2	1.2×10^{-6}
2	2×10^8	3×10^3	1.5×10^{-5}
3	10^9	1.8×10^3	9×10^{-6}
4	8×10^8	4.6×10^2	2.3×10^{-6}
5	8×10^7	3×10^3	1.5×10^{-5}
6	4×10^8	3.1×10^4	1.5×10^{-4}
7	8×10^8	1.2×10^2	6×10^{-7}
8	10^9	1.2×10^3	6×10^{-6}
9	8×10^8	1.5×10^3	7.5×10^{-6}
10	10^9	1.3×10^3	6.5×10^{-6}

To 1 ml exponential growth phase YP medium culture of strain S 168 ind$^-$, 1 ml phage of preparation (m. o. i. = 0.33) was added. After 20 minutes incubation at room temperature the suspension was centrifuged for 10 minutes then washed with MM medium. After resuspending in 1 ml MM medium, each dilution was plated on 3 gGM plates. The number of transductants was counted after 48 hours at 37° C.

complex. During the multiplication of cells derived from transductants, viruses containing bacterial chromosomal material may be produced in varying numbers.

Transduction of the indole marker to various 168 ind$^-$ strains. In view of the cultural differences among various 168 ind$^-$ strains, their ability to act as recipients was checked. When tested with one IFT preparation of phage 3 NT, the 3 cultures behaved similarly (Table VIII).

The 3 suspensions obtained and used under the same conditions, yielded transductants at an approximately uniform proportion. In colonial morphology, however, the transductants differed according to the properties of the parent strain.

Transduction of the indole marker by different phages. Comparison of phages 3 NT, PPS 1 and SP 10 was performed with phage preparations obtained

Table VIII

Transduction of the indole marker to different 168 ind$^-$ strains

Medium	No. of transductants per ml of recipient suspension		
	S 168 ind$^-$	T 168 ind$^-$	L 168 ind$^-$
gGM	8.7×10^3	9.4×10^3	1.5×10^4
MM	1.1×10^4	8.4×10^3	1.2×10^4

Titre of phage, 8×10^8 PFU/ml.

on donor strains NRS 231 and W_{23}. These strains were all sensitive to the above phages. Uniform results were obtained in several repeated tests. One of these experiments is presented in Table IX.

It is seen that phages 3 NT and PBS 1 propagated on strain NRS 231 transduced the indole marker at a frequency lower than that observed with phage SP 10. Preparations made on strain W_{23} yielded similar results. The

Table IX

Transduction of indole allele to strain S 168 ind⁻ by phages propagated on strain NRS 231

Phage	Phage titre PFU/ml	m. o. i.	No. of transductants/ml bacterium+	No. of transductants/PFU
3 NT	1.2×10^9	1.6	20	2.5×10^{-8}
PBS 1	1.4×10^9	1.6	40	5×10^{-8}
SP 10	4×10^9	1.6	500	6.2×10^7

+ Suspension S 168 ind⁻ contained 5×10^8/ml colony forming units. Phage-treated bacteria were plated on gGM agar.

alternative experiment was not performed, because phage SP 10 could not be propagated on the Yale strain or on its derivatives.

It should be noted that bacteriocins of strains W_{23} and NRS 231 were similar, but differed from those of strain Yale or its derivatives, and, in contrast to the latter, W_{23} and NRS 231 could be lysogenized with phage SP10. These findings may explain the fact that on strains W_{23} and NRS 231 phage SP 10 produced a more active transducing phage material than the PBS 1-type phages did.

Stability of prophage 3 NT

It has been observed that the lysogenic complexes of strain Yale and phage 3 NT are unstable and during the multiplication of lysogenic bacteria the prophage is not transferred to the majority of derivatives. The following experiment throws light on this finding. An exponential growth phase culture of strain Yale was infected with phage 3 NT (m. o. i. = 0.3), then incubated. The supernatant of a sample taken in the stationary phase contained 10^9 PFU/ml. The centrifuged bacteria were treated with anti-phage serum, then the numbers of colony and infective centre forming units were determined. As the respective values were 1.6×10^8/ml and 7.5×10^7/ml, only about 50 per cent of the cells contained prophages. On YP agar two colonial types were observed: some colonies were similar to those produced by the original, non-lysogenic bacteria; others were smaller with a more glistening and convex

surface (Fig. 1). The latter colonies consisted of lysogenic cells, the former colonial type contained non-lysogenic cells sensitive to phage 3 NT. The suspension of lysogenic colonies was treated with anti-phage serum and plated. Colonies

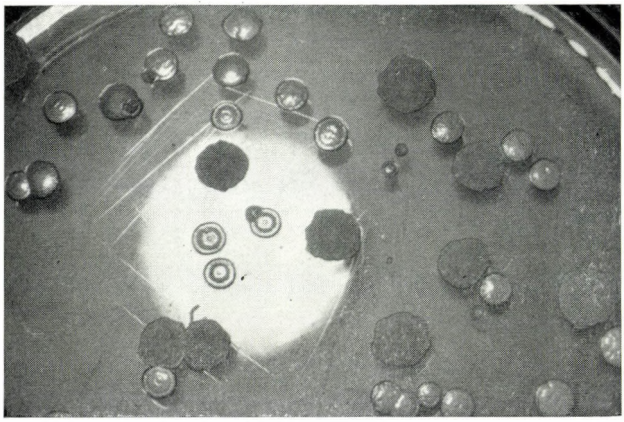

Fig. 1. Colonies obtained after anti-phage serum treatment from a stationary phase Yale culture infected with phage 3 NT. Large flat colonies: non-lysogenic; small glistening colonies: lysogenic cells. Magnification × 1.5

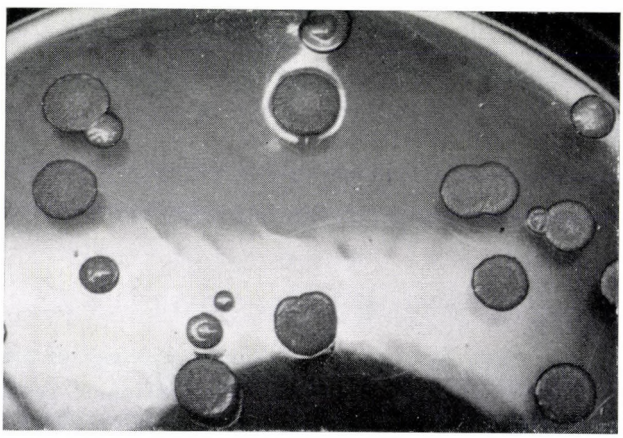

Fig. 2. Subculture of lysogenic clones (see Fig. 1) after anti-phage serum treatment. The sector in one of the colonies contained lysogenic bacteria. Magnification × 1.6

growing from this culture were lysogenic in 30 per cent. The loss of prophage was associated with a change in colonial morphology. Sometimes colonies with sectorial parts containing lysogenic bacteria were produced (Fig. 2). Cells within the sector were lysogenic. On further subcultivation of such cells, the loss of prophage was even higher. After multiple subcultures the incidence of lysogenic colonies was only one or two per cent.

Non-lysogenic segregants were sensitive to phage 3NT. Therefore the finding was explained with the loss of a whole phage genom and not with defectiveness.

Table X

Co-transduction of his⁻ derivatives of strain 168 ind⁻ by phage 3 NT

Strain	Co-transduction
SB 25 ind⁻ his⁻	+
13 ind⁻ his⁻	+
31 ind⁻ his⁻	+
26 ind⁻ his⁻	—
30 ind⁻ his⁻	—
SBI ind⁻ his⁻	—

Comparison of co-transduction by phages 3 NT and SP 10

EPHRATI—ELIZUR and FOX [9] demonstrated a linkage of indole and some histidine alleles in transduction experiments with phage SP 10. In our studies we observed the co-transduction of his⁻ derivatives of strain 168 ind⁻ (Table X).

Co-transduction by phage 3 NT propagated on the prototrophic Yale strain is in accordance with data of the gene map of EPHRATI—ELIZUR *et al.* [10], who showed in transformation experiments the location of the histidine linkage group. The his⁺ property could be transferred to histidine loci situated near to the ind⁻ locus (25; 13; 31). On the other hand, in the case of more distant loci (26; 30; SBI) no co-transduction was observed.

The co-transduction frequency of the indole and histidine loci by phages 3 NT and SP 10 was compared by the use of auxotroph 31 ind⁻ his⁻. Phage preparations were obtained on strains Yale and W_{23}. After phage treatment, the bacteria were plated on gGM agar containing 25 μg/ml indole or 25 μg/ml histidine. The obtained transductants were replica plated on gGM agar. Pooled data of several experiments are presented in Table XI.

Co-transduction was observed at considerable frequencies by both phages. In our experiments the transduction frequency by phage SP 10 was 7 to 10 per cent higher than the value given by EPHRATI—ELIZUR and FOX [9]. The high co-transduction frequency yielded by phage 3 NT differs significantly from the results obtained with phage SP 10. This finding may be due to a difference between the two donors or between the properties of the two phages.

Table XI

*Frequency of co-transduction of indole and histidine markers to recipient strain 31 ind⁻ his⁻**

Phage	Distribution of transductants on selective media			
	gGM + indole		gGM + histidine	
	ind⁺ his⁺	ind⁻ his⁺	ind⁺ his⁺	ind⁺ his⁻
3NT	1068 (98.2%)	19	1142 (98.7%)	25
SP10	980 (76.8%)	298	727 (82.5%)	155

* Pooled data of several experiments. After phage treatment strain 31 ind⁻ his⁻ (Yale donor) was plated on gGM medium; the colonies were replica plated on gGM medium.

Transduction of ammonium assimilation

As described above, S 168 ind⁻ populations contained two kinds of cell. The majority of the stock culture consisted of cells capable of growing on a medium containing ammonium salt as a sole source of nitrogen. In addition to these Am⁺ cells, the rest of the population produced colonies only on media containing glutamic acid, aspartic acid or asparagine (Am⁻ bacteria). Am⁻ cells survived in stock cultures of strain 168 ind⁻, which had been maintained in our laboratory as well as previously in other laboratories on complete medium (yeast extract peptone agar). When Am⁻ bacteria were cultured in liquid YP medium, towards the end of the exponential phase the population contained Am⁺ cells at a ratio of approximately 10^{-6}. In order to show whether ammonium assimilation is determined by chromosomal or some extrachromosomal factor, we have attempted to transduce this property by use of phage 3NT.

As compared to S 168 ind⁻ Am⁺, the ammonium negative strain can be regarded as a double auxotroph (ind⁻ Am⁻). The phage material of a lysogenic ind⁺ prototroph obtained with phage 3NT from strain S 168 ind⁻ Am⁺ was used for the treatment of an S 168 ind⁻ Am⁻ population. The treated bacteria were plated on the following media: (1) gGM + 25 μg/ml indole, which supported the growth of all bacteria; (2) MM + indole, which selected ind⁻ Am⁺ cells; (3) gGM agar, which selected ind⁺ Am⁻ transductants; (4) MM, which supported the growth of cells only when a co-transduction of indole and ammonium markers had occurred. The results are shown in Table XII.

It is seen that on gGM + indole medium phage-treated and control suspensions produced equal numbers of colonies. When strain S 168 ind⁺ Am⁺ was used as donor, on MM + indole plates the phage-bacterium mixture containing 2.6×10^8 cells/ml, formed only 10^5 colonies, in other words transduction of the Am⁺ locus was successful in 0.04 per cent of the population. Transduction frequency in that case was 3.2×10^{-4}/PFU. As indicated by the

Table XII

Transduction of ammonium locus (Am) by phage 3NT

Number of colony forming units on different media in suspensions treated and not treated with phage

Agar plate	No. of colony forming units/ml suspension			
	Phage donor			
	S 168 ind$^+$ Am$^+$		L 168 ind$^+$ Am$^+$	
	Phage-treated	Control	Phage-treated	Control
gGM + indole	2.6×10^8	2.9×10^8	2.5×10^8	2.7×10^8
MM + indole	1×10^5	2.6×10^2	1.2×10^4	2×10^2
gGM	4×10^3	0	5.3×10^3	0
MM	0	0	0	0

Strain S 168 was plated on gGM + indole medium. After 48 hours at 37°C, besides larger (4—5 mm) pigment-producing colonies, some small (1 mm), colourless colonies developed. An exponential growth phase YP medium culture of the latter colonies was infected with phages produced by lysogenic transductant S 168 ind$^+$ Am$^+$ treated with phage 3 NT. The bacteria were plated on different media. Suspensions not treated with phage served as controls. In case of strain L 168 ind$^+$ Am$^+$, the 3 NT phage material was obtained from lysogenic bacteria of the corresponding culture. M. o. i. values were 0.5 to 1.

number of colony forming units on MM + indole plates (2.6×10^2/ml), suspension ind$^-$ Am$^-$ contained Am$^+$ back mutants at a ratio of 10^{-6}. Transduction of the indole locus itself was of a lower frequency, as the phage-treated bacteria gave only 4.2×10^3 colonies per ml on gGM medium (1.4×10^{-5}/PFU)

The results were similar when ammonium-utilizing strain L 168 ind$^+$ Am$^+$ was used as donor. In these experiments, too, transduction of the Am locus occurred at higher frequencies than that of the the indole locus. Co-transduction of the two loci was not observed in either experiment.

Discussion

Since ROMIG and BRODETSKY [11] had published the first systematic study on subtilis phages, a number of investigators became interested in this group of bacterial viruses. Most of the phages so far described and studied had been isolated by use of the Marburg strain [1, 7, 11, 18]. In contrast, THORNE isolated phage SP 10 on *B. subtilis* strain W_{23}. We have been unable to obtain data as to the exact origin of strain W_{23}. Our studies indicate that, on the basis of phage sensitivity and "bacteriocin" production, it differs from the strain of BURKHOLDER and GILES [6] and from its derivatives. The "bacteriocins" are probably identical with the incomplete phages described by SEAMAN *et al.* [8]. It would appear that incomplete PBSX prophages vary

with different *B. subtilis* strains. Some of our observations indicate that differences in sensitivity to subtilis phages may be determined by these defective prophages.

The main points of our results have already been discussed in the experimental part of this paper. It should, however, be emphasized again that our transducing phages are similar to, or identical with, phage PBS 1. One of the examined *B. subtilis* strains, NRS 231, is uniformly sensitive to these phages and to the otherwise different phage SP 10. For the titration of temperate subtilis phages strain NRS 231 is very valuable not only owing to its sensitivity but also because of the well-defined plaques obtainable with this culture.

It is known that the nitrogen requirement of many strains belonging to the genus Bacillus can be supplied with ammonium salts. This also holds true for *B. subtilis*. One of our laboratory type cultures, strain S 168 ind^- split off cells unable to utilize ammonia. The main pathway of ammonia assimilation is the amination of a pyruvate by alanine dehydrogenase [12]. It, appears that in Am^- mutants transamination from alanine to ketoglutaric acid is blocked [12]. Our Am^- mutant did not utilize alanine and some other amino acids, but was able to use glutamic and aspartic acids. The latter substances are both important amino group donors in transamination. Ammonia assimilation could be transduced by phages to the Am^- mutant. Therefore, the Am^- and Am^+ properties are presumably allelic.

In our comparative studies we have also examined the high lability of prophages of bacteria lysogenized by phage 3 NT. The loss of prophage occurred at a frequency approximately similar to that found by TAKAHASHI [4] for phage PBS 1. It should be noted that segregation of the prophage has been definitely confirmed also on the basis of morphological difference between lysogenic and non-lysogenic colonies.

The unstable connection of prophage to the bacterial chromosome is seemingly common in certain lysogenic systems of the genus Bacillus. Such findings have been described for a temperate anthrax phage [13] and for other lysogenic systems in *B. subtilis* [11]. TAKAHASHI [14] explained the instability of the prophage by a difference between the DNA composition of phage PBS 1 and *B. subtilis*. It would appear that, in contrast to enteric bacteria, the connection between the prophage and the host cell in the genus Bacillus is often very loose. Further studies on phages of the genus Bacillus may reveal new uncommon DNA structures in addition to those already described [15—17].

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β-GALACTOSIDASE ACTIVITY OF SACCHAROMYCES FRAGILIS

II. MODE OF ACTION

By

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Summary. Treatment of *Saccharomyces fragilis* cells with sodium pyrophosphate (Na–P–P) has been found to increase β-galactosidase activity. Enzyme activity is also increased by other factors influencing the metabolism, and by enzyme poisons such as potassium cyanide, sodium fluoride, sodium azide, and uranyl nitrate. If Na—P—P and sodium azide are added together, the effect on enzyme activity is additive. The increased enzyme activity after Na—P—P treatment is not reversed or inhibited by any of 20 different enzyme inhibitors except phenyl-mercury-acetate.

There is a correlation between β-galactosidase activity and the quantity of water-extractable phosphate at 0°C. Factors causing an increase in the water-extractable phosphate also increase β-galactosidase activity. Manganese increases β-galactosidase activity by enhancing phosphate concentration in the cells. A non-specific increase in cell permeability is also suggested after Na—P—P treatment. The possible mechanism of action of Na—P—P is discussed.

In previous experiments [5] it has been shown that the β-galactosidase activity of *Saccharomyces fragilis* was increased by sodium pyrophosphate (Na—P—P) treatment, while the activity of an acetone powder of the yeast decreased rapidly after treatment with Na—P—P.

Na—P—P did not disrupt the organisms and there was no fall in viable count after treatment. If after 60 minutes incubation activity returned to its original level, it could again be increased by a second treatment with Na—P—P. The reversible change in β-galactosidase activity could be repeated several times. The Na—P—P treated organisms behaved differently from untreated cells towards metal ions, *e.g.* zinc ions inactivated acetone powders but activated Na—P—P treated cells. The fact that Na—P—P is a chelating agent does not explain its mode of action since other chelating agents such as versene and oxine did not increase enzyme activity in intact cells.

The present experiments had the aim to gain information about the mechanism by which Na—P—P increases the β-galactosidase activity of whole cells of *Saccharomyces fragilis*.

Materials and methods

The organism and medium used in this work have been described previously [5]. Cell density was measured at 700 mμ using a Beckman DU spectrophotometer. *β-galactosidase activity* was determined as described previously. Radioactivity measurements were as described earlier, using a "64" Utility Scaler type 1871, System K. F. K. I.

Radioactive phosphorus taken up from disodium hydrogen phosphate was measured in the following way. The incubation mixture with the yeast was filtered through Oxoid membrane filters (2.0 cm) washed thoroughly with water, and counted directly after drying the membrane under an infrared lamp. In certain experiments (noted in the text) radioactive phosphorus taken up was measured by centrifuging the incubation mixture and sampling the supernatant.

The effect of enzyme inhibitors upon the subsequent stimulation of enzyme activity by Na—P—P treatment was determined in the following way. The cells were first incubated for 30 minutes at 25°C with enzyme inhibitor dissolved in 0.005 *M*, pH 7.0 Tris buffer. The cells were then centrifuged and incubated at 0°C for 20 minutes in Tris buffer containing 10^{-2} *M* Na—P—P. After this treatment the β-galactosidase activity of the cells was determined. Control tubes were run for all substances before and after Na—P—P treatment.

O-Nitrophenyl-β-D-galactoside (ONPG) uptake was measured by incubating with ONPG the *Saccharomyces fragilis* cells at 0°C. After centrifugation, ONPG concentration in the supernatant was determined by sodium hydroxide hydrolysis in a boiling water bath for 10 minutes. The concentration of ortho-nitrophenol was measured at 420 mμ.

Orthophosphate was determined according to TAUSSKY and SHORR [6], and *monosaccharides* by NELSON's [3] method. Influx and efflux of monosaccharides were determined according to BURGER *et al.* [1]. Chemicals were of analytical grade. ^{32}P was supplied as $Na_2H^{22}PO_4$ by the Hungarian Institute for Isotope Distribution.

Results

Experiments were designed to answer the following questions:

(1) Is there a connection between the increased β-galactosidase activity after treatment with Na—P—P and other metabolic processes?

(2) Is there a correlation between phosphorus metabolism and β-galactosidase activity?

(3) Does permeability for ONPG and other substances increase as a result of Na—P—P treatment?

Relationship of cell metabolism to β-galactosidase activity. To answer the first question, various enzyme inhibitors were examined in order to discover whether previous treatment with them was able to prevent the stimulation of enzyme activity by Na—P—P treatment. It can be seen from Table I that NaF, EDTA, uranyl nitrate and KCN do not prevent the increase of enzyme activity brought about by Na—P—P treatment, and, furthermore that they all increase β-galactosidase activity by themselves. NaF especially had this stimulatory effect. Na—P—P and enzyme poisons have an additive effect in increasing β-galactosidase activity in *Saccharomyces fragilis* living cells. The decreased activity of the acetone powder of *Saccharomyces fragilis* after Na—P—P treatment was not influenced by azide.

In addition to those listed in Table I, the the following substances were also examined: oxine (10^{-2} *M*), hydrazine-sulphate (10^{-2} *M*), thiosemicarbazide (10^{-2} *M*), dimethylglyoxim (10^{-2} *M*), sodium citrate (10^{-2} *M*), chloramphenicol ($2 \cdot 10^{-3}$ *M*), dimedon (10^{-2} *M*), thiourea (10^{-2} *M*), aureomycin (10^{-3} *M*) and terramycin (10^{-3} *M*). Phenyl-Hg-acetate was the only substance which inhibited the increased enzyme activity caused by Na—P—P.

β-galactosidase activity and phosphorus metabolism in Saccharomyces fragilis. In previous experiments, among those tested Na—P—P was the only

Table I

Effect of various inhibitors upon increased β-galactosidase activity of Saccharomyces fragilis

Compound	Concentration (*M*)	Activity μ*M*/mg/hour		Treated/untreated
		Before	After	
		Na—P—P treatment		
H_2O	—	0.19	0.44	2.3
NaN_3	10^{-2}	0.28	0.66	2.3
$CaCl_2$	10^{-2}	0.28	0.59	2.1
NaF	10^{-2}	0.42	0.62	1.4
EDTA	10^{-2}	0.23	0.44	1.9
Na-diethyl-dithio-carbamate	10^{-2}	0.28	0.66	2.3
Uranylnitrate	10^{-3}	0.32	0.515	1.5
Phenyl-Hg-acetate	10^{-3}	0.09	0.07	0.7
KCN	10^{-2}	0.30	0.53	1.7

1.16 mg dry weight of *Saccharomyces fragilis* cells was incubated for 30 minutes at 25°C in a mixture of 1 ml 0.005 *M* pH 7.0 Tris buffer + 1 ml compound and 1 ml distilled water. After centrifugation, enzyme activity was determined. Parallel tubes were treated by 10^{-2} *M* Na—P—P in Tris buffer for 20 minutes at 0°C.

phosphorus-containing substance which at constant concentration and pH activated the β-galactosidase of *Saccharomyces fragilis*. In the present experiments we have examined factors known to influence the phosphorus metabolism for their effect on the β-galactosidase activity of the cells.

On comparing *Saccharomyces fragilis* cultures stored for various periods of time it was found that fresh cells had a higher enzyme activity than cells stored at 0°C. The activities could be correlated with the quantity of orthophosphate extractable from the cells by either Tris buffer or distilled water (Table II).

Table II

Duration of storage, enzyme activity, and quantity of phosphate released from Saccharomyces fragilis cells

Culture	Activity μ*M*/mg/hour	P released μg/mg dry weight/20 minutes
Fresh	1.62	77
Stored 24 hours at 0°C	1.18	35

Cells were centrifuged and suspended in 5 ml 0.01 *M* pH 7.6 Tris buffer. P was determined in 2 ml samples of the supernatant

Table III

β-galactosidase activity of Saccharomyces fragilis cells incubated for 20 minutes in phosphate buffer at 0°C

Compound	Concentration	Activity μM/mg/hour
H_2O		0.82
K-phosphate buffer pH 6.8	2×10^{-2}	0.78
	4×10^{-2}	1.05
	5×10^{-2}	0.94
	10^{-1}	1.45

Table IV

^{32}P uptake by Saccharomyces fragilis cells in the presence of Mn^{++} ions

Incubation mixture (M)				Radioactivity counts/min. (Corrected values)	
				0 min.	30 min.
K-phosphate		0.6×10^{-1}	pH 6.8	160.4	304.0
Glucose	+	5×10^{-2}			
K-phosphate		0.6×10^{-1}	pH 6.8	8314.4	9934.4
Glucose	+	2.5×10^{-2}			
$MnSO_4$	+	1.6×10^{-3}			

1.0 mg dry weight of *Saccharomyces fragilis* cells was incubated in the above mixtures (total volume, 1.0 ml). After mixing at 0° C a sample of 0.5 ml was taken and put upon membrane filter, then washed with 5.0 ml ice-cold distilled water. Mixtures were incubated at 25°C for 30' before second sampling.

The enzyme activity of cells stored at 0°C could be increased by the presence of orthophosphate (10^{-1} M) but to a much smaller extent than by Na—P—P (Table III). Although ROTHSTEIN *et al.* [4] working with *Saccharomyces cerevisiae* found no increased phosphate uptake in the presence of Mn^{++} ions, we have shown that phosphate uptake (or retention?) is increased by Mn^{++} in *Saccharomyces fragilis* (Table IV). Accordingly, we have examined the effect upon β-galactosidase activity of Mn^{++} and potassium phosphate added separately or in combination. It can be seen from Table V that β-galactosidase activity of *Saccharomyces fragilis* cells incubated for 60 minutes in potassium phosphate buffer containing Mn^{++} ions increased up to twofold an effect similar to that of Na—P—P treatment. From the previous experiments [5] it is known the increase of β-galactosidase activity depends upon the concentration of phosphate and Mn^{++}. The presence of an enzyme activator is also sug-

Table V

Enzyme activity of Saccharomyces fragilis cells after incubation in phosphate buffer and Mn^{++} ions

Incubation mixture	Concentration (M)	Activity μM/mg/hour
H_2O (0°C)		0.89
K-phosphate (pH 6.8)	10^{-1}	1.01
	1.4×10^{-1}	1.16
	2.0×10^{-1}	1.34
$MnSO_4$	0.33×10^{-2}	0.99
$MnSO_4$ +	0.33×10^{-2}	
+K-phosphate	6.6×10^{-2}	1.76
Glucose (in H_2O)	10^{-1}	0.54
Glucose +	10^{-1}	
+K-phosphate+	6.6×10^{-2}	2.13
+$MnSO_4$	0.33×10^{-2}	

1 mg dry weight/ml of cells was incubated for 60 min. at 25°C, in mixtures listed in the Table. After centrifugation the supernatant was sucked off, the cells were suspended in H_2O, and enzyme activity was determined as described under Methods.

gested by the shape of the curves obtained by plotting enzyme activity against cell concentration (Fig. 1) [2]. Phosphate by itself might play an activiting role. Both phosphate uptake (or retention?) and β-galactosidase activity are enhanced by Mn^{++}. However, $^{54}Mn^{++}$ uptake is not increased after Na—P—P treatment (unpublished results). A role of some cell constituent in the activa-

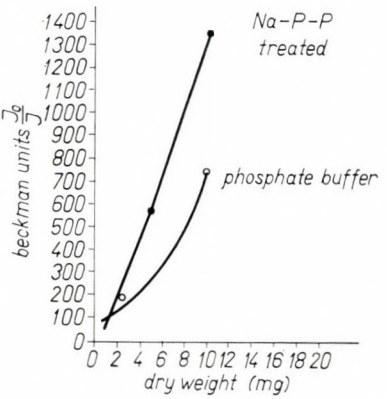

Fig. 1. β-galactosidase activity of control and Na-P-P treated *Saccharomyces fragilis* cells at different cell concentrations

Table VI

Enzyme activity of Saccharomyces fragilis clones before and after Na—P—P treatment

Number of clones	Activity μM/mg/hour		
	Initial activity	Incubated at 0° C in H_2O	Incubated at 0° C in Na—P—P
1	0.66	0.66	1.53
2	1.2	1.5	2.5
3	1.8	1.67	6.1
4	2.1	2.01	1.7
5	1.1	1.17	2.42
6	1.49	1.77	3.12
7	5.6	4.3	13.6
8	4.1	3.4	7.1
9	1.8	2.1	6.1
10	1.12	1.12	2.12
11	13.2	13.8	27.1
12	1.1	1.35	2.05
13	2.02	2.47	4.35
14	1.35	1.35	1.76
15	1.13	0.91	1.87
16	0.81	1.01	1.58
17	0.88	1.73	2.6

Saccharomyces fragilis was grown on solid media (the liquid medium with 2 per cent agar-agar added, individual colonies were cut out, suspended in distilled water, centrifuged and resuspended in distilled water or Na—P—P. After incubation and centrifugation the supernatant was sucked off, the cells were suspended in H_2O and enzyme activity was determined as described under Methods.)

tion process is also indicated. The increase of enzyme activity after Na—P—P treatment varies much with individual *Saccharomyces fragilis* clones (Table VI) and there is a limiting cell concentration below which there is no increase in enzyme activity after Na—P—P treatment.

Glucose, which in the absence of phosphate decreases the β-galactosidase activity of *Saccharomyces fragilis* cells, does not inhibit the activation elicited by the presence of Mn^{++} and phosphate (Table V).

Both sodium azide and 2,4-dinitrophenol even at low concentrations increased the β-galactosidase activity of *Saccharomyces fragilis* in the presence of phosphate but not in its absence. The results with sodium azide are shown in Table VII.

VII

β-galactosidase activity of Saccharomyces fragilis cells after incubation in azide and phosphate buffer

Incubation mixture	Concentration (*M*)	Activity μM/mg hour
H_2O		0.39
Azide (in Tris buffer)	0.33×10^{-1}	1.32
Azide (in Tris buffer)	0.16×10^{-1}	0.66
Azide (in Tris buffer)	0.08×10^{-1}	0.42
K-phosphate	0.66×10^{-1}	0.42
K-phosphate +	0.66×10^{-1}	1.65
+Azide	0.33×10^{-1}	
K-phosphate +	0.66×10^{-1}	1.03
+Azide	0.16×10^{-1}	
K-phosphate +	0.66×10^{-1}	0.66
+Azide	0.08×10^{-1}	

1.0 mg dry weight/ml of cells was incubated for 30 minutes at 25° C in the above mixture. After centrifugation the supernatant was sucked off, the cells were suspended in H_2O, and enzyme activity was determined.

The β-galactosidase activity of *Saccharomyces fragilis* rapidly increased in the presence of Mn^{++} and phosphate and an additional increase occurred during a further period of incubation. The addition of sodium arsenate (0.6×10^{-3} *M*) did not prevent the initial increase in activity or the subsequent increase during incubation at 25° C (Table VIII).

Permeability. ^{32}P was used to examine phosphate uptake by *Saccharomyces fragilis* and phosphate concentration in the supernatant decreased more in cells treated with Na—P—P than without such treatment (Table IX). When phosphate uptake was measured by ^{32}P activity remaining in the cells, results indicated a lower phosphate retention (Table X). It seemed advisable to measure the phosphate content of cells grown in the presence of ^{32}P after various treatments. Cells harvested from ^{32}P containing media retained various levels of radioactivity after treatment with Na—P—P, Na_2HPO_4 or distilled water. The most marked decrease of radioactivity was noted after Na—P—P treatment. It follows that the amount of phosphate which can be washed out by ice-cold distilled water was increased (Table XI).

It did not seem probable that selective permeability was increased by Na—P—P treatment, because treated cells took up or retained a smaller

Table VIII

β-galactosidase in Saccharomyces fragilis cells after treatment with arsenate and phosphate ions

Compound	Concentration (*M*)	Activity μM/mg dry weight/hour after incubation for	
		0 min.	60 min
K-phosphate pH 6.8	6×10^{-2}	1.42	
K-phosphate pH 6.8 +	6×10^{-2}	1.42	
+Sodium arsenate	0.6×10^{-3}		
K-phosphate +	6×10^{-2}	1.73	
+Sodium arsenate	0.3×10^{-3}		
K-phosphate +	6×10^{-2}	2.07	2.09
$+Mn^{++}$	3×10^{-3}		
K-phosphate +	6×10^{-2}		
$+Mn^{++}$ +	3×10^{-3}	1.9	3.01
+Sodium arsenate	0.6×10^{-3}		
K-phosphate +	6×10^{-2}		
$+Mn^{++}$ +	3×10^{-3}	1.9	3.07
+Sodium arsenate	0.3×10^{-3}		

1.2 mg dry weight *Saccharomyces fragilis* cells were incubated in volumes of 3 ml of the above mixtures. After incubation, and centrifugation the supernatant was sucked off, the cells were suspended in H_2O and enzyme activity was determined as described under Methods.

quantity of TMG than control cells [5]. We have determined arabinose and galactose transport by means of the method of BURGER *et al.* [1], but influx and efflux did not change significantly after Na—P—P treatment. It was necessary, however, to examine specifically the ONPG uptake by *Saccharomyces fragilis* after Na—P—P treatment. Experiments have shown that cells treated with Na_2HPO_4 or Na—P—P took up the same quantity of ONPG, but more than control cells treated with distilled water (Table XII).

The increase was higher than would be expected from calculation of the water-space if there is uniform distribution of ONPG in the cells. This result was explained by the results shown in Table XIII. The enzyme activity of *Saccharomyces fragilis* is not decreased by lowering the incubation temperature to 4°C to the extent that could be expected from VAN T'HOFF's law. From Table XIII it can be seen, too, that increased enzyme activity after Na—P—P treatment does not take place at 4°C if activities are determined by values extrapolated to zero time.

Table IX

^{32}P uptake of Saccharomyces fragilis cells after Na—P—P treatment

Treatment	No. of tubes	Activity in supernatant counts/min.		Per cent
		Corrected values	Mean	
Blank (no cells)	1	17.872	17.656	100
	2	17.440		
H_2O	1	17.240	17.076	96.7
	2	16.912		
Na—P—P	1	16.525	16.464	93.3
	2	16.404		

The cells were treated with H_2O or (10^{-2} *M*) Na—P—P at 0°C for 20 min. To 27.3 mg dry weight of cells was added 2.8 ml of a ^{32}P containing mixture (260 μg P/ml). The suspensions were kept at 0°C for 20 minutes, then centrifuged. Radioactivity measurements were done in 0.5 ml samples of the supernatant.

Table X

^{32}P uptake by Saccharomyces fragilis cells pretreated in distilled water or in Na—P—P

Treatment	Activity counts/min. (Corrected values)
Distilled water	122.4
Na—P—P	98.4

Cells were pretreated at 0°C for 20 min. in distilled water or Na—P—P (10^{-2}). After centrifugation the cells were incubated for 30 minutes at 25°C with ^{32}P a mixture generally used for determination of ONPG activity (in 1 ml of a 4 : 1 mixture of 0.2 *M* K-phosphate and ONPG). Radioactivity was determined from 0.5 ml samples pipetted on membrane filter and washed with 5.0 ml ice-cold distilled water

Table XI

Radioactivity of Saccharomyces fragilis cells cultivated in ^{32}P containing media, after Na—P—P treatment

Treatment	Radioactivity of cells counts/min. (Corrected values)	Per cent
H_2O	68.3	100
Na_2HPO_4	57.3	84
Na—P—P	48.4	71

Saccharomyces fragilis cells were cultivated in ^{32}P (3157 counts/min.) containing media for 48 hours. Cells (0.98 mg/tube) were centrifuged, the supernatant was discarded, and the residue was incubated in 1.0 ml volumes of H_2O, Na_2HPO_4 and Na—P—P ($10^{-2}M$) for 20 minutes, at 0° C. Cells were centrifuged and then incubated in a 1 : 2 mixture of K-phosphate (0.2 *M*) and H_2O for 30 minutes, at 25° C. Cells were pipetted on membrane filter and washed with 5.0 ml ice-cold water. Radioactivity was measured together with the membrane filter.

Table XII

ONPG uptake by Saccharomyces fragilis cells at 0°C

Treatment		ONPG uptake μg/ml dry weight
H_2O		5.7
Na_2HPO_4	10^{-2} *M*	10.0
Na—P—P	10^{-2} *M*	10.0
Toluol	2 per cent	10.0

Saccharomyces fragilis cells (19.6 mg dry weight) were treated for 20 min. at 0° C in the listed mixtures. After centrifugation the cells were suspended and incubated for 15 min. at 0°C in 2.0 ml of a mixture of 5.0 ml 0.2 *M* K-phosphate + 2.5 ml of ONPG (6 mg/ml) + 7.5 ml H_2O. ONPG concentration in the supernatant was determined by estimating ONP after hydrolysis with 0.14 *N* NaOH.

Table XIII

β-galactosidase activity of Saccharomyces fragilis cells at various temperatures

Duration (minutes)	Activity			μM/mg dry weight/hour		
	Na_2HPO_4 treatment			Na—P—P treatment		
	4° C	18° C	28° C	4° C	18° C	28° C
5	0.80	1.43	2.57	0.77	5.3	7.85
15	0.31	1.01	1.47	0.47	3.39	6.24
30	0.165	0.55	1.28	0.33	3.32	5.43
60	0.178	0.723	1.22	0.31	3.74	4.58

Discussion

In the above experiments the mode of action of Na—P—P treatment in increasing β-galactosidase activity of *Saccharomyces fragilis* has been investigated.

As a general rule, decrease of the metabolism of freshly harvested cells by enzyme-inhibitors or by low temperature was followed by an increase of β-galactosidase activity and an enhanced effect of Na—P—P treatment. No substance has been found which would have significantly inhibited the increased enzyme activity caused by Na—P—P treatment. The effect of phenyl-Hg-acetate cannot be considered specific from this point of view.

The increased enzyme activity disappeared during incubation of the cells in distilled water. This return to the original level of activity is speeded up by the presence of glucose. The further addition of phosphate buffer prevented this decrease of activity.

It has not been possible to measure directly the permeability of the cells to ONPG. The water space could not be calculated by means of the

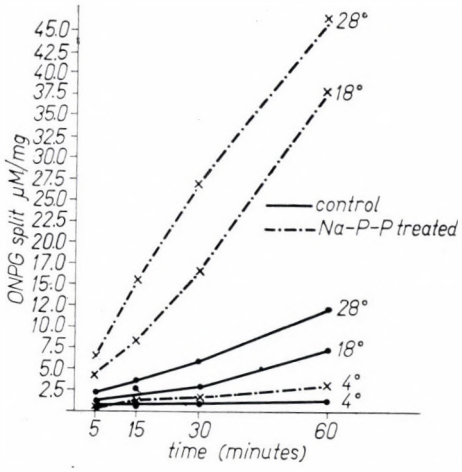

Fig. 2. β-galactosidase activity of control and Na-P-P treated cells at various incubation temperatures

values of ONPG disappearing from the supernatant. ONPG was split at 0°C and the quantity of ONPG which had disappeared from the supernatant was bigger than was expected if it had been uniformly distributed in the water space.

A non-specific increase of permeability of cells was indicated by the following facts: the release of 260 mμ absorbing material was increased after Na—P—P treatment, the phosphate and TMG retaining capacity decreased and the activity of another enzyme, β-glucosidase, was also increased. (Unpublished results.)

The phosphate concentration in the supernatant of the suspension medium decreased to a lower level when the cells were pretreated with Na—P—P, but this increased uptake was not followed by a higher concentration of incorporated phosphorus. Only the amount of water-extractable phosphate in the *Saccharomyces fragilis* cells increased as a result of Na—P—P treatment.

An increase of water extractable phosphate would be produced if Na—P—P acted as uncoupling agent. The increased enzyme activity after NaF, azide and dinitrophenol treatment could be explained also by an inhibition of the processes of oxidative phosphorylation.

The question arises whether it is a general rule that all uncoupling agents cause a reversible increase of cell-permeability by inhibiting the processes of oxidative phosphorylation. The reversibly increased permeability of the cells can be regarded as an adaptation following inhibition of oxidative phosphorylation by toxic agents. The increased permeability would then help to eliminate the toxic substances from the cells more quickly, and the original permeability is restored when inhibition of oxidative phosphorylation has ceased.

The possibility cannot be excluded that some labile derivative of phosphate rather than phosphate ions acted as activator.

A further possibility still remains. Our experiments do not exclude, and the curves of enzyme activity at various temperatures render it possible, that Na—P—P activates a permease system responsible for ONPG uptake. The lack of activation at 0°C and that of a lag phase in ONPG splitting at 18°C in the case of cells treated with Na—P—P in contrast to control cells can be cited in support of such a hypothesis (Table XIII and Fig. 2).

In conclusion, the following hypothesis would explain the action of Na—P—P treatment on the β-galactosidase activity of *Saccharomyces fragilis*. Na—P—P acts as an uncoupling agent and the consequent disturbance of oxidative phosphorylation gives rise to increased permeability, while phosphate, or some other activator of β-galactosidase, accumulates within the cells. This hypothesis would explain the fact, too, that the effect of Na—P—P is not specific for β-galactosidase.

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POSSIBLE CAUSES OF LEUCINE INHIBITION IN ESCHERICHIA COLI K_{12} λ-28

By

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Summary. Leucine inhibits the growth of *E. coli* K_{12} λ-28, by lengthening the lag-phase. This inhibition is increased by purine and pyrimidine bases, and decreased by their antagonists. Treatment with chloramphenicol of washed cells in the presence of leucine increased the leucine-sensitivity of the cells. These findings suggest the possibility of RNA synthesis playing a role in the inhibition caused by leucine.

ALFÖLDI *et al.* [1, 2] have demonstrated that, in contrast to stringent strains, the growth of relaxed *E. coli* strains on complete medium could be inhibited by amino acids in the course of plating on minimal medium. This inhibition could be suspended by combinations of amino acids. Considering the different behaviour of relaxed and stringent strains it has been assumed that RNA metabolism participated in the inhibitory effect. In a previous paper [3] the amino acid sensitivity of certain strains originating from *E. coli* K_{12} was examined by a method different from that applied by ALFÖLDY *et al.* [1, 2]. It was found that the inhibiton did not depend on the presence of a certain allel of the RC locus and, on the other hand, the stringent strain of *E. coli* K_{12} λ-28 proved to be the most sensitive to several amino acids. Considering that in our experiments inhibition was found to be caused by the same amino acids and prevented by similar combinations of amino acids as in the experiments of ALFÖLDI *et al.*, it was assumed that the mechanism of inhibitions was similar. ALFÖLDY *et al.* [1] ascribed these effects to the differences between the stringent and relaxed strains in the first place. It seemed therefore necessary to prove the role of RNA synthesis from different aspects.

Material and methods

Strain. The prototroph strain of *E. coli* K_{12} λ-28 RC^{str} was used throughout.

Experimental conditions. Bacteria grown for 16 hours on complete medium were inoculated into the same medium. After two hours incubation the cultures were washed and transferred into DAVIS and MINGIOLI's minimal medium [4]. Growth was followed by determining the viable count. Further details have been described previously [3].

Results

Effect of purine and pyrimidine bases and their ribosides. Studying the effect of purine and pyrimidine bases upon the growth of *E. coli* K_{12} λ-28

in the presence of leucine, it was observed (Fig. 1) that 250 μg/ml of uracil in leucine-free medium affected growth only slightly, whereas, when with the addition of 2.5 μg/ml of leucine the lag-phase was prolonged to 12 hours, uracil increased it further to 20 hours. Similar results were obtained with cytosine, guanosine and adenosine. No significant effect could be attained with adenine. Owing to its weak solubility, guanine was not tested.

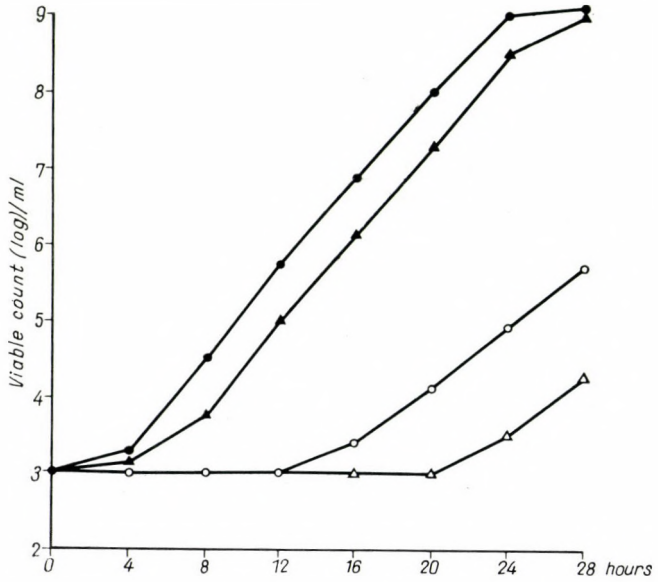

Fig. 1. Effect of uracil on leucine-sensitivity. Circles, control; triangles, 250 μg/ml uracil; filled symbols, leucine-free medium; empty symbols, medium containing 2.5 μg/ml of leucine

Effect of purine and pyrimidine antagonists. On applying antagonists which did not inhibit growth on complete medium, a substantial decrease in leucine-sensitivity occurred. In Fig. 2 the effect of 250 μg/ml of 5-amino-uracil is demonstrated. On leucine-free medium no considerable change in cell multiplication was noted; the 12-hour lag-phase observed in the presence of 2.5 μg/ml leucine decreased to 8 hours.

Similar results were obtained with 6-methyl-uracil and with the adenosine antagonists prepared by VARGHA and KUSZMAN [5] in this institute (α-chlor-adenosine, β-chlor-adenosine, α-epoxy-adenosine) (Fig. 3). No similar effect could be achieved with 6-aza-guanine, 6-aza-uracil, 5-Fl-uracil and 5-Br-uracil.

Effect of chloramphenicol (CAP). Sub-bacteriostatic doses of CAP had no effect on leucine-sensitivity. When 10^5/ml washed bacteria were incubated in minimal medium containing 20 μg/ml of CAP at 37°C for two hours, and then the

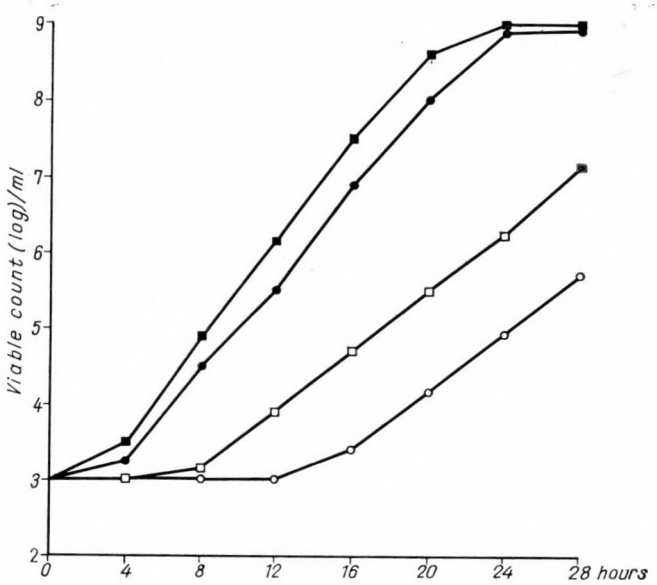

Fig. 2. Effect of 5-amino-uracil on leucine-sensitivity. Circles, control; squares, 250 μg/ml 5-amino-uracil; filled symbols, leucine-free medium; empty symbols, medium containing 2.5 μg/ml of leucine

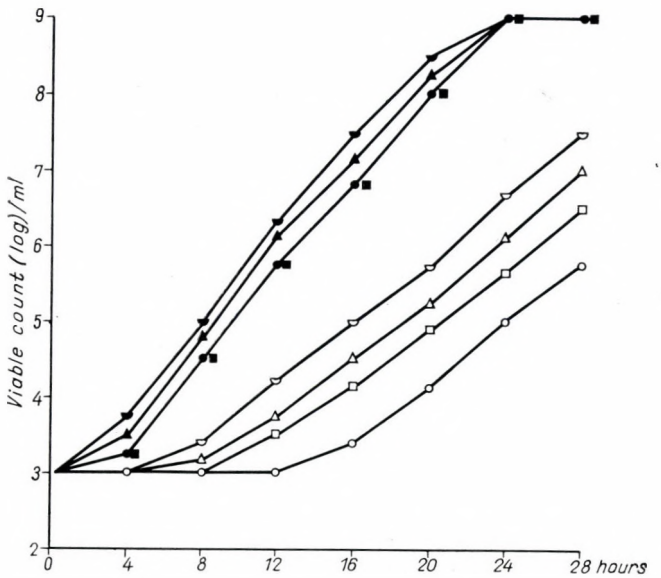

Fig. 3. Effect of adenosine antagonists on leucine-sensitivity. Circles, control; squares, α-chlor-adenosine; triangles, β-chlor-adenosine; half-circles, α-epoxy-adenosine. (The concentration of all antagonists was 250 μg/ml). Filled symbols, leucine-free medium; empty symbols, medium containing 2.5 μg/ml of leucine

effect of CAP was interrupted by diluting 1 : 100, leucine-sensitivity did not change as compared to non-treated cells. Leucine inhibition was, however, significantly enhanced when besides CAP 2.5 μg/ml of leucine had been present during preincubation and dilution had been made into a medium containing similar amounts of leucine. In this case the lag-phase was lengthened by at

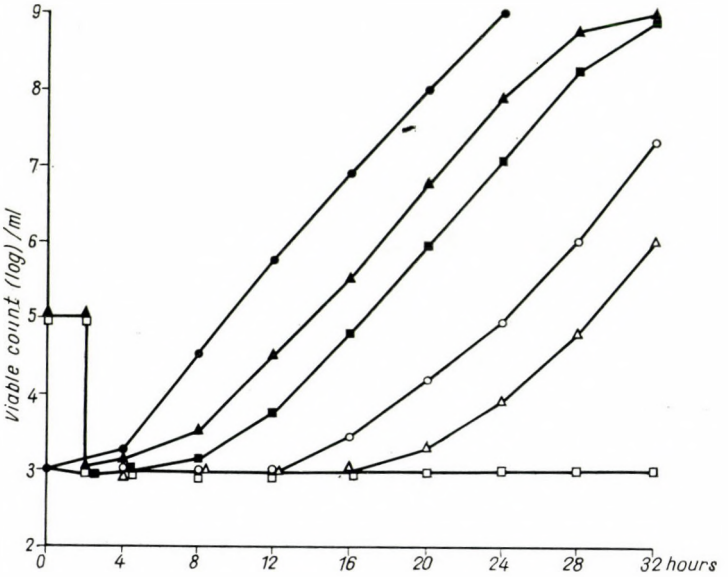

Fig. 4. Effect of chloramphenicol on leucine-sensitivity. Circles, without preincubation; triangles, preincubation with 2.5 μg/ml CAP, squares, preincubation in the presence of 20 μg/ml CAP and 2.5 μg/ml leucine; filled symbols, leucine-free medium; empty symbols, medium containing 2.5 μg/ml of leucine

least 20 hours, in contrast with the moderate prolongation recorded on leucine-free medium (Fig. 4).

This phenomenon could not be observed with cells grown in minimal medium.

Discussion

Our results with purine and pyrimidine bases support the hypothesis of ALFÖLDI *et al.* that the regulation of RNA synthesis plays a role in the inhibition caused by amino acids. It can be assumed that by influencing the rate of RNA synthesis they increase or decrease the amount of RNA analogous with the relaxed RNA formed in a stringent strain in the presence of leucine in the course of the shift down period.

It is known that owing to the effect of CAP, a protein-deficient so-called CAP-RNA arises [6]. This CAP-RNA is not functioning, and after the removal of CAP protein synthesis starts only with a delay [7]. The relaxed RNA forming in the course of amino acid starvation of relaxed strains reminds in composition and functioning of the CAP-RNA [8]. ALFÖLDI and KEREKES [2] assume that leucine increases the accumulation of relaxing RNA in a relaxed strain through the stimulated synthesis of a catholic inducer. Our present observation of the synergetic effect of the combination of leucine and CAP suggests that also in the case of stringent strains leucine inhibits growth through the RNA metabolism.

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A NEW COMPONENT FROM THE CELL WALL OF STREPTOMYCES GRISEUS

I. THE ROLE OF STREPTOMYCIN IN THE LIFE OF STREPTOMYCES GRISEUS

By

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Summary. In order to obtain experimental data on the role of streptomycin in the life of the strain which had produced it, the mycelia of a *Streptomyces griseus* mutant strain which had lost the capacity to excrete streptomycin into the medium, have been analyzed. The cell wall of this strain was isolated and analyzed to find the constituents of the streptomycin molecule.

Streptidine has been isolated and identified and streptomycin (or streptidine) is considered a cell wall constituent of the mutant *Streptomyces griseus* strain not producing streptomycin.

Thousands of substances with antibiotic properties have been found in the last two decades and several of them have been defined structurally. It is supposed that they are the products of the microbial cell metabolism derived "from the main metabolic pathway of protein, fat, carbohydrate and purine metabolism" [1]. There is, however, no generally accepted explanation for the role of antibiotics in the life of the organisms producing them.

Krassilnikov [2] considers these substances as tools in the biological fight among various species evolved in the past history of the relevant microbe. According to Waksman and his school [3], antibiotics are waste products of the micro-organisms and are produced by chance. A third theory, Levitov's [4], — concerning penicillin production — suggests detoxicating mechanisms in the micro-organism as the basis of antibiotic production.

In order to elucidate the role of streptomycin in the life of the strain producing it, we have compared some biochemical and morphological characteristics of a streptomycin-producing *Streptomyces griseus* strain and some streptomycin-non-producing *Streptomyces griseus* mutants [5, 6]. From these results it has been concluded that the streptomycin-non-producing mutant strains have a shorter life cycle characterized by the appearance of thick-walled reproductive hyphae. The development of the thick wall is preceded by the accumulation of periodic acid-Schiff (PAS) positive substances in the hyphal wall [7]. The cell wall of the streptomycin-non-producing mutant showed a higher pentose content than that of the streptomycin-producing *Streptomyces griseus* strain [8, 9].

Since streptomycin is a carbohydrate-containing compound excreted into the medium by the producing strain, it was supposed that the streptomy-

cin-non-producing strain, if it synthetized streptomycin, might build the molecule into some cell constituent. In the following experiments we were looking for the components of streptomycin in the mycelia of a streptomycin-non-producing *Streptomyces griseus* mutant strain.

Materials and methods

Strains, media and cultivation. The origin, characteristics, and cultural behaviour of the *Str. griseus* strains, and the composition of the soy-bean medium used in the experiments have been described previously [6].

Isolation and purification of the cell walls. The methods of isolation and purification of the cell walls in the experiments has been described previously [8, 9].

Detection of guanidine groups. Detection of guanidine-containing compounds was carried out by the modified SAKAGUCHI reaction [11].

Hydrolysis conditions. (a) *For carbohydrate analysis* the mycelia of the *Str. griseus* strains were harvested at appropriate time and washed five times with distilled water and hydrolyzed with *N* H_2SO_4 for four hours in sealed tubes at 100°C. The supernatant fluids were used for carbohydrate determination. (b) *For paper-chromatographic and infrared determinations and for purification by ion exchange resin* the cells were prepared as follows. Twenty mg of the walls of the streptomycin-non-producing strain were hydrolyzed with 2 ml of 12 per cent HCl in sealed ampoules at 100°C for three hours. The hydrolysate was evaporated to dryness and dissolved in distilled water. This process was repeated three times, to eliminate the excess of HCl. The dry material was dissolved in distilled water and the insoluble parts were separated by centrifugation. The supernatant was used for the determinations [12].

Isolation of the Sakaguchi-positive compound. The Sakaguchi-positive substance derived from the cell wall was isolated on a column of Amberlite IRC—50, in NA^+ form. Elution was done with *N* HCl. The eluted fractions were evaporated to dryness, the Sakaguchi-positive material was extracted from the dried elutions with absolute ethanol and evaporated to dryness. This final product (s) was used for analytical determinations.

Paper chromatography. For paper chromatography the following solvent systems were used: n-butanol-pyridine-water (3/2/3—V/V/V); n-butanol-acetic acid-water (73/10/17—V/V/V); n-butanol saturated with water; n-butanol-methanol-water-p-toluene-sulphonic acid (10/40/20/1—V/V/V/W) [13], and n-butanol-acetic acid-water (4/1/5—V/V/V) [14]. Whatman No 1 filter paper was employed in the descending system.

Carbohydrate determination. The carbohydrate content of the mycelia was determined by the anthrone reaction according to TAKASHITA [10].

Results

In the first orientating experiments the harvested and washed mycelia in submerged culture (Fig. 1) showed that the carbohydrate content of the mycelial hydrolysate of the streptomycin-non-producing strain reaches a higher final value, whereas the carbohydrate content of the producing strain, although higher initially, decreases with time. This decrease is parallel to streptomycin production.

On the basis of these results and considering the high carbohydrate content of the cell wall [8, 9], and the fact that the streptomycin molecule is composed of carbohydrates, we have tried to find the constituents of the streptomycin in the cell wall hydrolysate of the non-producing *Streptomyces griseus* strain. Isolation and purification of the cell wall was carried out as described earlier [8, 9].

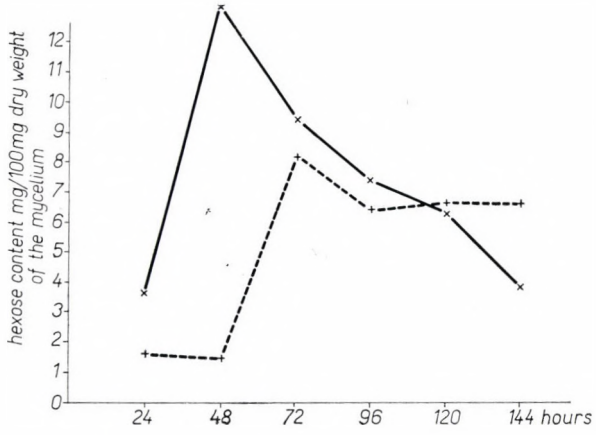

Fig. 1. Carbohydrate analysis of mycelia
×——× Mycelial hydrolysate of the streptomycin-producing strain × — — × Mycelial hydrolysate of the streptomycin-non-producing strain

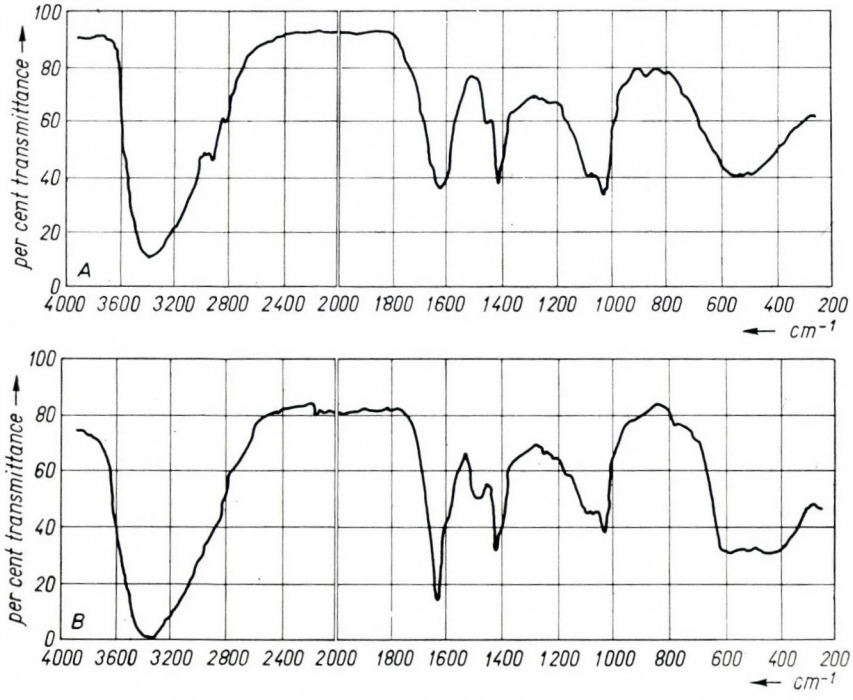

Fig. 2. The isolated and purified unknown substance derived from the cell wall. The streptidine control was treated as the unknown material. The compound were used for measurement in KBr pastilles.
Fig. 2A: infrared spectrum of streptidine control. Fig. 2B: infrared spectrum of substance isolated from cell wall

The cell wall hydrolysates were examined by paper chromatography in five various solvent systems (as described under Materials and methods). The spots were developed by the Sakaguchi reaction [11]. One of the substances had the same R_f value as the streptidine-sulphate applied as control.

This compound from the cell wall hydrolysate was isolated on Amberlite IRC— 50 column. The isolated substance showed the same paperchromatographic characteristics in all the five solvent systems and had the same infrared spectrum than that of streptidine-hydrochloride (Fig. 2).

Arginine being a Sakaguchi-positive substance, we had to exclude the possibility of its identity with our Sakaguchi-positive compound. Comparing the infrared spectra of streptidine, arginine, and our substance, it became evident that the spectrum of arginine differed from that of streptidine and of our substance. This fact was supported by paperchromatographic results.

We have not succeeded in distinguishing our material from arginine on the basis of the ultraviolet spectrum at different pH values.

Discussion

Our results indicate that the streptomycin molecule may play a role in cell wall structure. The streptomycin-producing strain would excrete the streptomycin molecule into the medium while the non-producing mutant would build it into the cell wall. This is also suggested by the high carbohydrate content of the streptomycin-non-producing strain demonstrated in Fig. 1. The decreasing carbohydrate content of the streptomycin-producing strain is explained by the excretion of the carbohydrate-containing antibiotic, the streptomycin.

It remains to be proved whether streptomycin itself or its constituents take part in building up the cell wall structure. Further investigations will be needed to elucidate this question.

Acknowledgements. We are indebted to Mrs. J. BREUER (Research Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest) for taking the infrared spectra, and to K. MAGYAR and I. HORVÁTH (Research Institute of the Pharmaceutical Industry, Budapest) for supplies of streptidine-sulphate.

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ISOLATION OF CYTOMEGALOVIRUS AND INCIDENCE OF COMPLEMENT-FIXING ANTIBODIES AGAINST CYTOMEGALOVIRUS IN DIFFERENT AGE-GROUPS

By

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(Received November 2, 1964)

Summary. A group of 16 symptom-free premature babies and infants with clinically demonstrated cytomegalic inclusion disease or interstitial pneumonia has been examined. Urine samples from 4 infants yielded cytopathogenic agents in human embryonic fibroblast cell cultures. On the basis of their growth characteristics, cytopathogenicity and neutralisation tests, the isolated agents appeared to be cytomegaloviruses (CMV). The properties of the strains are described. In spite of failing serology, the typical clinical picture and the successful isolations suggested the possible aetiological role of the isolated agents.

The incidence of CF antibodies against CMV was found to increase with age in serum samples obtained from a total of 442 persons of different ages. Titres of 1 : 4 or higher were present in 18, 28, 35 and 51 per cent in the age groups of 2 to 24 months, 4 to 14, 14 to 30 and over 30 years, respectively.

Incidence and titres of CF antibodies against CMV in the sera of 60 mentally defective children corresponded essentially to the data obtained in healthy children of the same ages.

During recent years several reports have been published on the isolation of CMV from humans. Successful isolations were reported by Smith [1] from salivary glands, by Rowe [2, 3] from adenoids and urine, by Weller [4] from liver biopsy samples of patients with cytomegalic inclusions disease, and by Hanshaw [5], Medearis [6] and Stern [7] from urine.

The studies presented below have been performed in order to isolate the agent from healthy babies or from those with typical clinical symptoms, and for determination of the incidence of specific CF antibodies in sera of persons of different ages.

Materials and methods

Virus isolation was attempted from a total of 16 newborn babies and infants either healthy or displaying clinical symptoms of cytomegalic inclusion disease.

A modification of the method of Medearis [6] was used for the pretreatment of urine samples serving for virus isolation. The urine specimen was centrifuged and the supernatant was diluted with an equal amount of nutrient medium (45 per cent Parker's 199, 45 per cent Hanks' solution with 0.5 per cent lactalbumine hydrolysate, 10 per cent bovine serum, and antibiotics); pH of the mixture was adjusted to 7.2 by bicarbonate. Tubes of human embryonic fibroblast cultures were inoculated with 1 ml each of the inoculum prepared as described above. Adsorption was allowed to take place for 2 hours and then the inoculom was replaced by nutrient medium. Infected cultures were incubated at 37° C. The urine specimens were inoculated within 2 hours following collection.

Maintenance of isolates. When diffuse cytopathic changes had developed in the infected cultures, the cells were suspended by means of a scraper and 0.1 ml of this suspension was transferred to fresh cultures of human embryonic fibroblasts. The medium described above was used for both the nutrition and maintenance of the cultures. The medium was changed every 4 to 5 days.

Stained preparations. The cells were cultivated on coverslips and infected as described in the corresponding section. Indirect immunofluorescence tests were performed on the 4th to 5th day following infection [8]. Human convalescent serum was used as specific immune serum.

Neutralization test. The method suggested by VÁCZI *et al.* [9] for the neutralization of varicella-zoster virus was used. Infected cultures incubated for 9 to 10 days were trypsinized and the suspension was centrifuged. Resuspension of the sedimented cells was accomplished in serum-free PARKER's 199 solution in a volume equal to that used for the cultivation of the same cells. A tenfold dilution series was prepared of the cell suspension and each dilution was mixed with an equal volume of specific immune serum. Neutralization was allowed to take place for 2 hours at 37° C and then the suspension was diluted with PARKER's 199 medium so as to obtain a final serum concentration of 10 per cent. One ml of the different dilutions was transferred into 3 tube cultures each. The neutralization efficiency was expressed in per cent reduction of focus numbers referred to the control with normal calf serum. Neutralization was performed with a human serum of high complement fixing (CF) antibody titre. CF was positive in 1 : 256 dilution of the serum with a standard CMV strain (Virus Laboratory, St. George's Hospital Medical School, London) as antigen. No CF was obtained with varicella-zoster antigen, while with herpes simplex virus antigen a titre of 1 : 4 could be observed.

Immunization of rabbits. Immunization was performed with the standard CMV strain obtained from infected cultures of human embryonic fibroblast cells. After appropriate incubation the infected cells were suspended and washed three times in PBS. The final sediment was resuspended in PBS and homogenized by sonication (MSE ultrasonic apparatus; 1.5 A, 30 sec., 10 ml suspension, $^3/_4''$ titan head). The homogenisate was centrifuged, ampouled and stored at −20° C until used.

The amount of antigen administered to a rabbit during a complete course of immunization represented the disintegrate of a total of 2×10^7 infected cells. The antigen was administered in increasing doses twice weekly for 3 weeks. On the 4th week the animals were bled and those exhibiting satisfactory titres were exsanguinated. Sera were stored at −20° C.

Before use, each serum was subjected to 3 subsequent 30 min. cycles of absorption with 1.5×10^6 human fibroblast cells/ml.

Preparation of CF antigens. Sonically disintegrated 10 to 12 day old cultures of the appropriate virus on human fibroblasts were used as CF antigen.

The CF reaction was performed by TAKÁTSY's [10] micromethod with an overnight incubation at +4° C.

Results

Isolation. The clinical data of the 4 infants excreting cytopathogenic agents with the urine were, (1) B. J., newborn of 8 days. Thrombopenic purpura, hepatosplenomegaly, jaundice, kyphoscoliosis. (2) B. I., 6 months old infant. Interstitial- and bronchopneumonia. (3) S. T., infant of 5 months. Interstitial- and bronchopneumonia. (4) Sz. J., 2 days old newborn. Symptom-free.

Cultivation of the agents and characterization of their cytopathic effects. The early cytopathic changes observed after 2 to 14 days in infected cultures of human embryonic fibroblast cells consisted of foci of small round or enlarged oval, refractile cells. No multinucleated giant cells were present in the foci.

The number and diameter of the foci increased with time. The central cells underwent degeneration and after 25 to 30 days of incubation the foci were confluent.

The agents producing the above described changes could be maintained through consecutive passages. The time required for the development of specific cell damage decreased on repeated passages, being 15 to 17 days and

8 to 10 days in the first and later passages, respectively. In the first 5 passages cell suspensions were required for successful transfers while attempts with the fluid phase failed. On further passages the fluid phase also proved to carry the agent. Nevertheless, the infection produced by the latter resulted in a protracted development of typical cytopathic lesions.

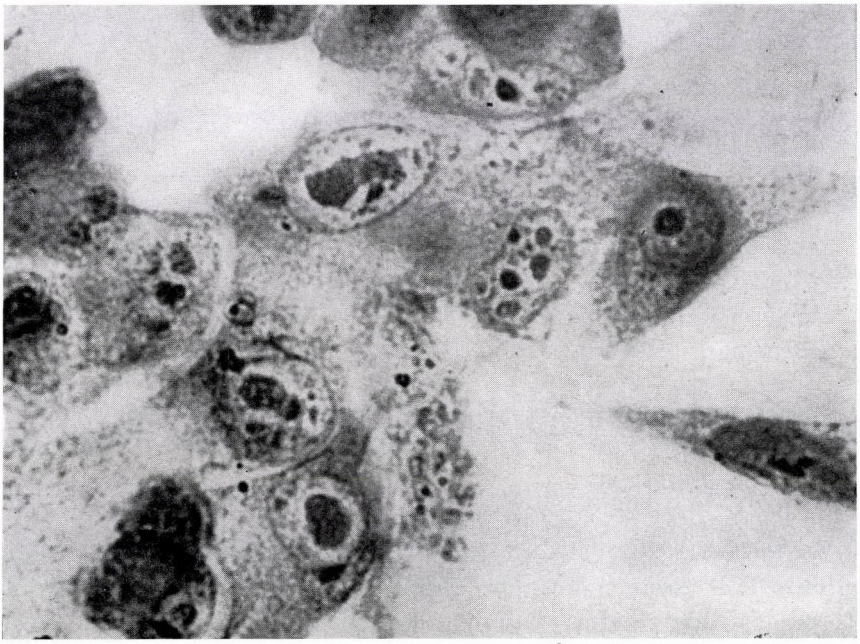

Fig. 1

In preparations stained with haematoxylin—eosin the infected cells appeared slightly swollen and formed small islands within the layer of normal fibroblasts. Typical A type eosinophilic inclusion bodies, 2 to 3 in number were present in the nuclei of the damaged cells. The inclusions were finely granulated and surrounded by a halo. In the cytoplasm no characteristic changes were demonstrable (Fig. 1).

The isolated agents were then examined by the indirect fluorescence method. Using high titre human convalescent sera, specific fluorescence could be detected in the cytopathic foci produced by all of the 4 isolated agents.

None of the agents grew on primary monkey or HeLa cells. All the 4 agents proved to be ether-sensitive.

Serology. Neutralization tests were performed with a serum of high specific CF activity selected from those collected for the serological survey of the population. As compared to the control, this serum reduced the focus-

forming activity of our CMV—1, CMV—2, CMV—3 and CMV—4 strains by 96, 94, 95 and 91 per cent, respectively (Table I).

Rabbit immune serum. CF reaction. The unadsorbed immune serum prepared in rabbits gave a positive CF with both infected and uninfected human embryonic fibroblast cell culture extracts at 1 : 2056 dilution. Adsorption with normal human embryonic fibroblast cells, as described above, reduced the homologous titre to 1 : 128, while adsorption antigen from infected cultures was essentially unaltered.

Table I

Neutralization of isolated strains by immune serum

Initials of patient	Strain isolated from patient	Virus titres* with		Neutralization capacity of immune serum against the isolated viruses
		calf serum	immune serum	
B. J.	CMV — 1	23	1	96%
B. I.	CMV — 2	48	3	94%
S. T.	CMV — 3	88	4	95%
Sz. J.	CMV — 4	63	6	91%

* Focus forming units/0.1 ml.

Neutralization test. The adsorbed immune serum produced a definite reduction of the focus count. This result could, hovewer, not be evaluated satisfactorily as the residual tissue specific antibody still had some cytotoxic effect.

CF with acute and convalescent sera of patients and antigens prepared from our isolates. The homologous CF antibody titres in acute and convalescent sera of babies yielding a cytopathogenic agent were examined. Results are given in Table II.

One of the babies (B. J.) exhibited identical CF antibody titres in both the acute and convalescent serum specimens. The mother of this baby had the same antibody titre in her serum. None of the further three babies had CF antibody in their acute or convalescent sera.

The incidence of CF antibodies against CMV was examined in healthy persons of different ages. The results shown in Table III revealed that the incidence of CMV specific CF antibodies increase with age. Titres of 1 : 4 or higher were observed in 18, 28, 35 and 51 per cent of persons of 2 to 24 months, 4 to 14, 15 to 30 or more years of age, respectively. The antibody content of the umbilical blood samples was about the same as that in adults.

CMV infection is known to cause mental defects [11, 12]. We examined therefore the incidence of CMV antibodies in a total of 60 mentally defective

Table II

CF test with the isolated virus strains

Initials of patiens	Strain isolated from patient	CF titres in		
		acute serum	convalescent serum	mother's serum
B. J.	CMV — 1	1 : 64	1 : 64	1 : 64
B. I.	CMV — 2	Negative	Negative	not tested
S. T.	CMV — 3	Negative	Negative	not tested
Sz. J.	CMV — 4	Negative	Negative	not tested

children. The data obtained were essentially identical with those found in normal children.

Table III

CF antibodies against CMV in different age groups
($\geqslant$ 1 : 4 regarded as positive)

Age groups	Number of sera tested	Positive	
		No.	%
Umbilical blood	88	41	47
2—24 months	34	6	18
4—14 years	120	33	28
14—30 years	100	35	35
> 30 years	100	51	51
Total	442	166	38
Mentally defective children			
4—14 years	60	17	29

Discussion

The isolated CP agents proved to be CMV. They multiplied in human embryonic fibroblast cell cultures, showed a characteristic CP effect with typical A type intranuclear inclusion, produced moderate amounts of extracellular virions and were neutralized by CMV specific immune sera.

The neutralization test with CMV was carried out as recommended by Váczi *et al.* [9] for varicella-zoster viruses. The method is based on the principle that the number of foci produced by the virus is reduced by specific

immune sera. The reason for applying this method to CMV was the fact that the virion/infectivity ratio of the virus was very high, between 10^7 and 10^8 [13].

The aetiological role of the agents isolated in our laboratory could not be ascertained satisfactorily. Only one (B. J.) of the babies with positive virus isolation exhibited homologous antibody in his paired sera. No difference was, however, demonstrable between the titres of the acute and convalescent samples. The mother of this baby had in her serum the same antibody of the same titre, thus the maternal origin of the baby's antibodies could reasonably be supposed. Repeated attempts to demonstrate CMV antibodies in the sera of the other three babies have failed, probably as these babies were prematures or severely ill and anergic, thus unable to produce antibody. Nevertheless there was no unequivocal evidence of the aetiological role of any of the isolated agents. Similar observations have already been published, for instance, HANSHAW [5] isolated CMV from the urine of 3 children with leucaemia and of 1 with Hodgkin's disease out of a total of 50 children with different tumours.

One of the isolates (CMV—4) was obtained from the urine of a symptom-free premature baby. ROWE [3] did not succeed in isolating CMV from throat washings of 108 healthy newborns.

On the basis of our recent studies on the incidence of CMV in premature babies these agents seem to play some aetiological role in premature births.

WELLER *et al.* [14] could not obtain specific antibodies by immunization of laboratory animals. The rabbits immunized by us developed high titre antibodies against CMV. After adsorption with normal human fibroblast cell disintegrates, the specificity of these antibodies was satisfactory in the CF test. This treatment failed, however, completely to eliminate the tissue specific antibodies, thus their cytotoxic effect rendered their use for neutralization tests impractical. Therefore all of our neutralization tests were performed with high titre human convalescent sera.

The incidence of antibodies against CMV in humans was found to increase with age.

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AETIOLOGY OF KERATOCONJUNCTIVITIS IN EPIDEMIC AND NON-EPIDEMIC PERIODS

By

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(Received November 5, 1964)

Summary. Systematic serological and virus isolation studies were performed during the keratoconjunctivitis epidemic (from December, 1961, to September, 1962) in Budapest and during the subsequent one and a half non-epidemic years. Virus isolation was attempted from a total of 51 clinical specimens collected during the epidemic. Out of them one yielded type 3 and four yielded type 8 strains of adenoviruses. Serological examinations (haemagglutination inhibition and complement fixation) have shown that in the epidemic period 63 per cent of the cases were caused by type 8 adenovirus.

Isolation experiments on 93 different specimens obtained from 81 clinical cases of keratoconjunctivitis observed in the non-epidemic period, yielded adenoviruses of different types (3, 4, 7, 14 and 16) and also Herpes simplex virus. The results of the isolation experiments were supported by serological testing. Type 8 of adenovirus caused but 15 per cent of the keratoconjunctivitis cases in the non-epidemic period.

In the years 1961—1962 Hungary experienced an epidemic of keratoconjunctivitis (EKC) [1]. Similarly to the results of aetiological studies performed in other countries, NÁSZ *et al.* [2], BÉLÁDI *et al.* [3], ÁGOSTON *et al.* [4] and FARKAS *et al.* [5] described type 8 adenovirus as the aetiological agent of the disease. Our examinations performed during the epidemic period yielded results supporting the above observations.

Our studies were conducted over an additional one and a half years (up to May, 1964) following the epidemic, to determine whether the sporadic clinical cases of EKC were caused also by type 8 adenovirus, the prevailing aetiological agent in epidemic cases.

The present paper is a report of the whole study including both the epidemic and non-epidemic periods.

Materials and methods

Specimens for virus isolation. The conjunctival and throat washings were collected at the 1st Department of Ophthalmology, University Medical School, Budapest; Department of Ophthalmology, Postgraduate Medical School, Budapest; and at the Department for Ophthalmological Diseases of the District XIV Outpatient Clinic, Budapest.

Isolation was attempted only from samples obtained during the first 5 days of the disease.

Sampling from the conjunctiva was made as follows. The conjunctival sac was rinsed with aliquots of antibiotic containing HANKS' solution, using a special pipette. An 0.1 ml amount of the undiluted fluid thus obtained was used as the inoculum. Throat washings were obtained as described earlier [6].

Virus isolation experiments. Each specimen was inoculated into 3 parallel tubes of 2 to 3 days old HeLa cell cultures. GEY solution [7], containing 5 per cent of inactivated rabbit serum and 0.25 per cent of lactalbumin hydrolysate was used as a maintenance medium. The culture media were renewed every 3rd or 4th day. The infected cultures were observed for a period of 23 to 28 days [3, 8]. No specimen was considered negative until 2 or 3 consecutive blind passages had turned out negative.

Standard virus strains. The prototype adenovirus strains of types 1, 2, 3, 4, 5, 6 and 7 were kindly supplied by Dr. D. BLASKOVIČ (Bratislava, ČSSR), whereas type 8 by Dr. I. BÉLÁDI (Szeged, Hungary). Types 11, 14, 15 and 16 of adenovirus were obtained by the courtesy of Dr. F. O. MACCALLUM (London).

Identification of the isolated agents was performed by neutralization tests using type-specific sera prepared in rabbits as described by DÖMÖK *et al.* [9]. For identification experiments an amount of 20 to 100 CPD_{50} of the appropriate virus was used. Mixtures of the virus and type serum were incubated for 2 hours at 37° C, then kept at +4° C overnight. Tubes of HeLa cell cultures were inoculated with 0.1 ml each of the above materials. Final reading was made on the 7th day.

Strains of Herpes simplex virus were identified by chorioallantoic inoculation, mouse pathogenicity and ether resistance tests.

Blood specimens. A total of 334 blood specimens were collected from 147 patients. Out of them 40 supplied 3 serum samples each. Serum samples obtained up to the 8th day following the onset of the disease were regarded as acute ones, while those taken 15 to 60 days later were designated as convalescent ones. All the specimens were inactivated at 56° C for 30 minutes before use.

Serological examinations. The complement fixation (CF) reaction was performed with TAKÁTSY's Microtitrator equipment, as described previously [10]. The antigens used in the CF tests were as follows. A mixture of different types (1, 4, 7 and 15) of adenovirus, inactivated at 56° C for 30 minutes and a native homogenate of chorioallantoic membranes infected with Herpes simplex virus.

Serum neutralization tests were performed using paired sera of 10 selected patients and 100 and 20 CPD_{50} virus from the types 3, 4, 14 and 8, respectively. Twofold serial dilutions were prepared from an initial 1 : 4 dilution of the test sera. The neutralization tests were carried out in tube cultures of HeLa cells, using 3 parallels per serum dilution. Final reading was made after 7 days of incubation.

Haemagglutination inhibition (HI) test. Haemagglutination and HI reactions were performed according to TAKÁTSY [11], using the Microtitrator equipment. For types 3, 7, 14 and 16 of adenoviruses rhesus red blood cells, whereas for type 8 human "O" erythrocytes were used. The inactivated sera were pretreated as described by ROSEN [12], in order to remove aspecific inhibitors or haemagglutinins. Twofold dilution series were prepared from sera in saline and to each dilution step 4 haemagglutinating units of virus were added, then they were incubated for 1 hour at room temperature. This was followed by adding the 1 per cent red blood cell suspension. After thorough mixing, the system was incubated at 37° C or at room temperature, depending on the type of virus used.

Results

Serological studies. A total of 147 paired sera were available from cases observed during the epidemic. Results of CF and HI tests performed with these paired sera and type 8 adenovirus are presented in Table I.

The number of positive reactions was remarkably higher in the HI than in the CF test and also the titre rises were greater in the former than in the latter. Out of the 77 paired sera giving a positive HI reaction, 31 exhibited a fourfold, and 22 a twofold, titre increase in the CF reaction. In the remaining 24 samples no increase of the CF titre was demonstrable. The HI reaction was negative in 4 cases out of those exhibiting a fourfold or higher titre increase in the CF test. These might be considered as EKC cases caused by an agent

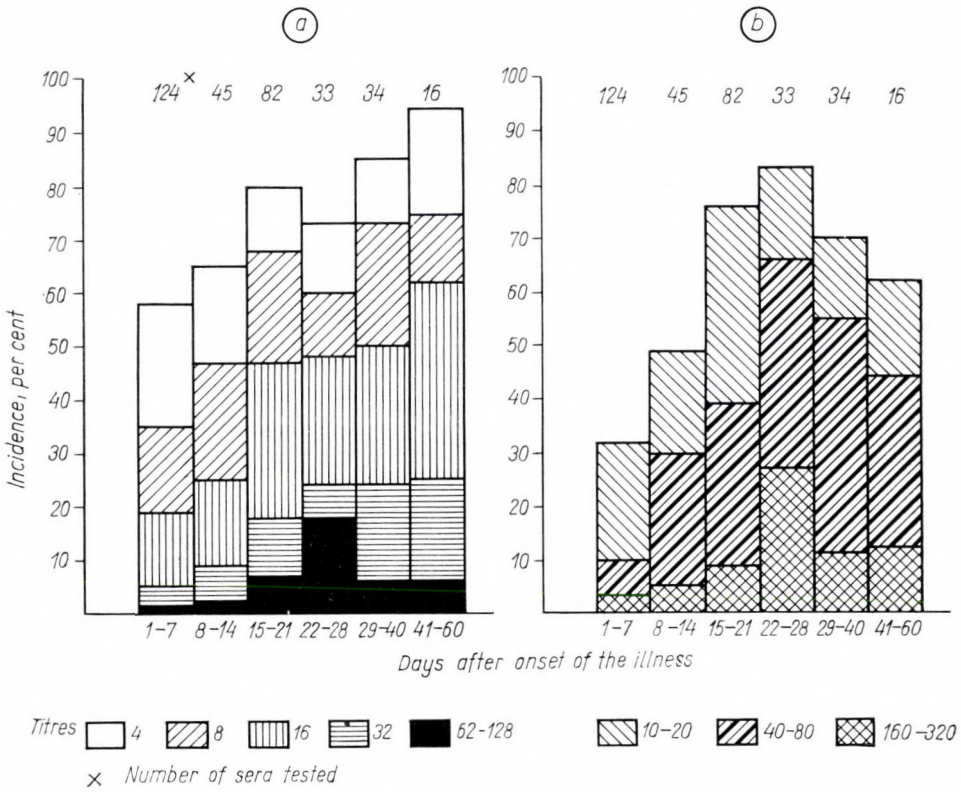

Fig. 1. Incidence of CF (a) and HI (b) antibodies against type 8 adenovirus in relation to the onset of illness

Table I

Comparison of the results of CF and HI tests obtained in cases of EKC

		Titre increase in CF*				Total	
		yes		no			
		No.	%	No.	%	No.	%
Titre increase in HI*	yes	31	21	46	31	77	52
	no	4	3	66	45	70	48
Total		35	24	112	76	147	100

* Fourfold or higher increase.

other than type 8 adenovirus. The sera of 15 patients exhibited a twofold titre increase in both the CF and the HI test, against type 8 adenovirus. In these cases the aetiological role of the agent appeared highly probable. Hence, if twofold increases of the HI titre were considered positive, the aetiological role of type 8 adenovirus was verified in 63 per cent of the examined cases.

The above results were checked by comparison to 108 paired sera taken at random for control purposes from non-EKC patients affected with other viral diseases. The HI tests were performed with type 8 adenovirus. None of

Table II

HI antibodies against type 8 adenovirus in the sera of patients suffering from EKC or from other viral infection

Patients suffering from		Number of examined cases	Number of patients with HI antibody titres*						
			< 10	10	20	40	80	160	320
EKC	No.	147	44	10	19	22	31	14	7
	%	100	30	7	13	15	21	9	5
Other viral diseases	No.	108	88	10	7	3	0	0	0
	%	100	81	9	7	3	0	0	0

* Reciprocals

the paired sera exhibited a fourfold titre increase; a twofold increase was demonstrable in two cases only. Thus, taking a twofold increase in HI antibody titre as a sign of positivity appears to be justified.

Table II shows the incidence of HI antibodies against type 8 adenovirus in the sera of EKC patients and control individuals affected with viral diseases other than keratoconjunctivitis. The data reveal a significant difference in the antibody levels of the two groups. In the control group, the highest HI-titre was 1 : 40 and even this level was attained in three cases only. None of these three persons had actually suffered from any type of ophthalmological disease. Out of the individuals having had EKC, only 30 per cent had HI titres lower than 1 : 10, whereas in the control group this category covered 81 per cent of the cases.

Fig. 1 presents the incidence of CF and HI antibodies against type 8 adenovirus at different points of time following the onset of EKC. In the CF-test, the occurrence of titre values above 1 : 64 reached a maximum level after 4 weeks, whereas the mean titres and the number of positive reactions

Table III

Keratoconjunctivitis cases yielding virus in the non-epidemic period

Case No.	Samples		Type of virus isolated	Duration of isolation experiment (days)	Serum +	Antibody titres**			
						homologous			adeno 8
	Kind	Time of taking*				CF	HI	N	HI
1.	Conjunctival washing	2	adeno 3	9	A C	<4 8	<10 160	16 1028	<10 <10
2.	Conjunctival washing	1	adeno 3	13	A C	<4 4	<10 20	— —	<10 <10
3.	Conjunctival washing	5	adeno 3	30	.	.	.	.	.
4.	Conjunctival washing	5	adeno 4	8	A C	<4 16	— —	64 256	<10 <10
5.	Conjunctival washing	5	adeno 8	84		.	.	.	.
6.	Conjunctival washing	1	adeno 14	7	A C	<4 8	— —	— —	<10 <10
7.	Conjunctival washing	5	adeno 14	19		.	.	.	.
8.	Conjunctival washing	3	adeno 14	8	A C	4 32	— —	— —	<10 <10
9.	Conjunctival washing Throat washing	2 2	adeno 14 adeno 14	14 13		. .	. .	. .	. .
10.	Throat washing	2	adeno 14	6		.	.	.	.
11.	Conjunctival washing	2	adeno 14	21		.	.	.	.
12.	Conjunctival washing	3	adeno 16	20	A C	4 32	<10 <10	— —	<10 <10
13.	Conjunctival washing	2	Herpes simplex	8	A C	<4 8	— —	— —	<10 <10
14.	Conjunctival washing	2	Herpes simplex	6	A C	<4 8	— —	— —	<10 <10
15.	Conjunctival washing	2	Herpes simplex	5	A C	<4 16	— —	— —	10 10

* Days after onset of disease.
** Reciprocals.
+A = acute phase; C = convalescent phase

showed an increasing tendency even after 6 weeks. In the HI test, however, both the number of positive reactions and the mean titre attained peak values after 4 weeks and later exhibited a gradual decrease.

The serological examinations yielded evidence of the aetiological role of type 8 adenovirus in the studied EKC epidemic. Negative serological tests were obtained with types 3 and 7 of adenovirus. This might be considered as an additional proof of the above finding. A twofold increase of titre against type 4 was observed in but one case. This case of EKC was observed at the beginning of the epidemic and the type 3 adenovirus was identified also by isolation. The virus neutralizing antibody titre of this patient exhibited a fourfold increase against type 3 adenovirus.

Virus isolation during and after the epidemic. During the epidemic period in Budapest a total of 51 specimens were examined. One of them yielded type 3, four of them type 8 adenovirus. In the latter cases the first cytopathic effect developed in the 2nd or 3rd passage, *i.e.* 40, 49, 50 or 57 days after inoculation into the tissue culture. In every case of positive isolation, the supposed aetiological role of the agent was further supported by the demonstration of an increase in the homologous neutralizing antibody level in the convalescent sera.

A total of 81 EKC patients was examined during the successive one and half year period (from October, 1962, to May, 1964). Both clinical specimens and paired sera were available in 49 out of the above cases. Specimens alone and paired sera alone were obtained from 24 and 8 patients, respectively. Thus a total of 93 clinical specimens (conjunctival and/or throat washings) and a total of 57 paired sera were examined. Positive isolations of adenovirus were made from 13 specimens obtained from 12 individuals. Three additional cases yielded Herpes simplex virus (Table III).

The aetiological role of the agent was demonstrated by both virus isolation and serological testing in 9 cases where both clinical specimen and paired sera were available. In all these cases, the CF and HI tests have confirmed the results of the isolation experiment. In none of the cases, however, could an increase of the HI titre against type 8 adenovirus be demonstrated. The HI test was performed with types 3, 7 and 8 of adenovirus using paired sera of 48 patients with negative or missing virus isolation experiments. There was an increase of the specific homologous titre against type 3 in two cases, against type 7 in one case, and against type 8 in eleven cases. In eleven additional cases the significant increase of the CF antibody titre suggested a history of infection with an unknown type of adenovirus. Negative CF and HI tests were obtained with 23 serum pairs against type 3, 7 and 8 antigens.

The results obtained in isolation and serological experiments may be summarized as follows. Out of a total of 81 patients 37 (46 per cent) yielded evidence of a previous adenovirus infection. The incidence of the different types in this group was 15 per cent for type 8; 17 per cent for types 3, 4, 7, 14,

and 16; and 14 per cent for other unidentified types of adenovirus. Herpes simplex virus was isolated in 4 per cent of the total. The aetiology of 50 per cent of the cases remained unknown.

Discussion

Based on the results of their extensive studies JAWETZ [13] and JAWETZ *et al.* [14—16] had reached the conclusion that the type 8 adenovirus is practically the only aetiological agent of EKC. This observation was later supported by a number of positive isolation and/or serological studies [17—21].

The epidemic of EKC experienced in Hungary in 1961 and 1962 was examined virologically by NÁSZ *et al.* [2], BÉLÁDI *et al.* [3], ÁGOSTON *et al.* [4] and by ourselves. The results of all these studies were in accordance with those mentioned above with regard to the aetiological role of type 8 adenovirus. In the epidemic period 63 per cent of our cases examined serologically was caused by this type. In all the cases with marked corneal symptoms the antibody titre against type 8 adenovirus was high [5].

In sporadic keratoconjunctivitis cases we demonstrated the aetiological importance of adenoviruses other than type 8 and of Herpes simplex virus. These results are in accordance with those of other authors [22—24], who demonstrated the aetiological role mainly of types 3 and 7 of adenoviruses and Herpes simplex virus. GRAYSTON *et al.* [25], examining an epidemic of EKC in Taiwan isolated a variety of different adenovirus types and demonstrated that typical corneal symptoms may be produced also by types 4 and 11. Our results have also suggested that type 8 adenovirus is not the sole agent of clinically typical keratoconjunctivitis. Thus the clinical picture alone does not permit definite conclusions concerning the aetiology.

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A NEW PHAGE TYPE OF STAPHYLOCOCCUS AUREUS ASSOCIATED WITH AN OUTBREAK OF PEMPHIGOID

By

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Summary. A staphylococcal phage type lysed only by a new phage has been described. The new phage, 42 D/1, was obtained by adapting phage 42 D to the causative agent of an outbreak of pemphigoid taking place in a newborn ward. During the 41 week observation period, of a total of 5689 nasal swabs, pemphigoid and faecal samples from infants, nasal swabs and milk samples from mothers, and nasal swabs from the staff, 3088 specimens were positive for staphylococci. Phage type 42 D/1 occurred in 44.7 per cent among the isolated strains. From pemphigoid specimens the new phage type was isolated in 81.3 per cent. Nasal swabs from infants with pemphigoid yielded this organism in 62.4, while those from infants without pemphigoid only in 39.2 per cent. The curve representing the incidence in carriers of the dangerous phage type showed several periodical peaks and declines. Independently of the total number of staphylococcal carriers, the number of pemphigoid cases was the lowest when the dangerous type was less frequent in carriers. Of the examined 1238 42 D/1 strains, 99.8 per cent were resistant to penicillin, 87.9 to streptomycin, 12.6 to chloramphenicol, 21.3 to tetracyclines, 3.4 to neomycin and 97.8 per cent to erythromycin.

In previous papers [1, 2] we have reported on a pemphigoid outbreak occurring during the summer of 1963 in a newborn ward, and on the hygienic measures applied for controlling the spread of infection. The present paper deals with micro-organisms responsible for the outbreak.

Materials and methods

Materials. Nasal swabs, pemphigoid and faecal specimens, mothers' milk, originating from newborn infants, mothers and staff, were examined. Altogether 5689 specimens from 814 persons were examined between August, 1963 and May, 1964.

Cultural methods. The materials were inoculated into SKORKOVSKI's broth containing 4 per cent potassium rhodanate [3], then plated on blood agar. Coagulase test was carried out by the slide method.

Phage typing was performed as described by WILLIAMS and RIPPON [4], with the following phages. Standard typing phages: 29, 52, 52A, 79, 80; 3A, 3B, 3C, 55, 71; 6, 7, 42E, 47, 53, 54, 75, 77, 83A; 42D; 81, 187. Additional standard phages: 42B, 47C, 52B, 69, 73, 78. Other phages: 2042, 2757, 13, 42 D/1, 756, 960. Typing with 2042, 2757, 13, 756 and 960 has been introduced partly in our laboratory, partly at the State Institute of Hygiene, Budapest.

Phage 42 D/1 used in the present work was obtained as follows. At the beginning of the outbreak it was obvious that most staphylococcal strains were not sensitive to routine typing phages (80—90 per cent from infants' noses and nearly 100 per cent from pemphigoid specimens). The untypable strains were practically uniform in antibiotic sensitivity (resistant to penicillin, streptomycin and erythromycin; sensitive to chloramphenicol, tetracyclines and neomycin). The strains were characterized by colonies producing strong yellow pigmentation and strikingly wide haemolytic zone. Thus it was probable that the cultures comprised one uniform organism not sensitive to routine typing phages.

Concentrated phages 79, 47 and 42 D gave weak aspecific reactions with our cultures. As the most definite reaction occurred with phage 42 D, using the soft agar method, we attempted to propagate this phage on one of the staphylococcal strains isolated from pemphigoid. A relatively high titre phage lysing the incriminated organism at 10^{-6} dilution was obtained. In lytic pattern the new phage differed from phage 42 D: it was inactive on strain 42 D, and in addition to lysing strain 42 D/1, it exerted some lytic action on strains 47 and 77. The new phage was provisionally named 42 D/1 and introduced in routine examinations. Staphylococci lysed by this agent were regarded as belonging to phage type 42 D/1.

Antibiotic sensitivity was determined by the use of penicillin, streptomycin, chloramphenicol, tetracycline, neomycin and erythromycin discs manufactured by the Institute for Serobacteriological Production and Research "Human", Budapest.

Results

First, the phage type distribution of staphylococci isolated from pemphigoid cases was determined. Pemphigoid occurred in 410 out of 814 infants either during their stay in hospital or after discharge. Bacteriological examination revealed staphylococci from 123 (54 hospital and 69 home) cases. The phage type distribution is presented in Table I.

Table I

Phage type distribution of 123 Staph. aureus strains isolated from pemphigoid specimens

Phage type	Pemphigoid manifesting		Total number of strains
	in hospital	after discharge	
42 D/1	46	54	100
Untypable	5	7	12
81	1	4	5
71	2	1	3
2042	—	2	2
75	—	1	1
Total	54	69	123

In Table I it is seen that the phage type distribution was fairly uniform among infections manifesting themselves before and after discharge from hospital. Of the 123 pemphigoid strains 100 (81.3 per cent) belonged to the new phage type 42 D/1; 11 (8.7 per cent) strains fell into four different phage types; and 12 (10.0 per cent) strains were untypable.

Phage group distribution of 3088 *Staph. aureus* strains isolated during the observation period is shown in Table II.

Table II

Percentage distribution among phage groups of 3088 Staph. au reus strains

Source	No. of strains	I	II	III	IV	M	Mixed	42 D/1	Other additional	Untypable
Nasal samples, infants in hospital	1225	7.1	2.9	6.5	—	0.4	2.7	55.5	0.9	24.0
Faecal samples, infants in hospital	213	2.8	—	16.0	—	—	—	57.3	0.9	23.0
Nasal samples, infants after discharge	342	6.7	2.7	7.9	—	—	—	49.4	2.9	30.4
Pemphigoid samples	123	4.0	2.4	0.7	—	—	—	81.3	1.6	10.0
Nasal samples, mothers in hospital	703	15.2	5.8	15.7	1.3	4.1	7.5	17.6	6.9	25.9
Milk samples, mothers in hospital	61	—	—	21.4	—	—	—	54.1	—	24.5
Nasal samples, mothers after discharge	233	12.9	5.1	13.4	—	1.6	2.1	35.3	5.6	24.0
Nasal samples, hospital staff	188	8.1	10.2	12.7	2.5	0.5	11.6	34.1	3.6	16.7
Total	3088	9.0	4.0	10.5	0.5	1.3	3.1	44.7	3.0	23.9

Among strains isolated from infants, phage type 42 D/1 predominated (55.5 per cent). Untypable strains occurred in 23.9 per cent; the remaining 19.6 per cent belonged to different groups. Phage type 42 D/1 and untypable strains were frequently isolated from mothers and staff. As to the total number of strains, the incidence of phage type 42 D/1 amounted to 44.7 per cent, that of untypable strains to 23.9 per cent. Members of 7 other phage groups occurred in 31.5 per cent. Fig. 1 shows the relative frequency of 42 D/1 strains in different materials.

In pemphigoid specimens phage type 42 D/1 was the most prevalent (81.3 per cent). In infants' nasal samples taken before and after discharge, in infants' stools and mothers' milk this organism occurred at similar frequencies (55.5, 49.4, 57.3 and 54.1 per cent, respectively). Among staphylococcal strains isolated from mothers' nasal samples while staying in the hospital, this phage type occurred in 17.6 per cent. This finding indicates that staphylococci harboured by mothers had mainly been acquired outside the hospital. It is interesting that 3 to 4 weeks after birth the same mothers were carrying phage type 42 D/1 in 35.3 per cent; in other words, after discharge from hospital, a number of the mothers acquired the epidemic strain from their own infants. The difference in the occurrence of the incriminated phage type between mothers' milk and nasal samples (54.1 vs. 17.6 per cent), was remarkable.

Especially during the first stage of the outbreak, phage type 42 D/1 was frequently revealed in nasal samples taken from the staff. Later these carriers were gradually removed to other departments and replaced by staff not harbouring the epidemic agent.

Of newborn infants with pemphigoid 62.4, of those without pemphigoid 39.2 per cent, harboured the incriminated organism.

Fig. 2 shows the incidence of nasal carriers among infants developing pemphigoid during and after their stay in hospital, and among infants who did not contract the disease.

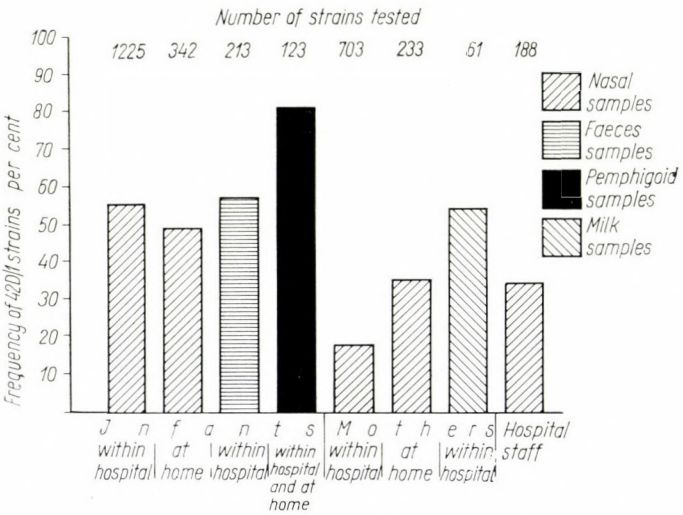

Fig. 1. Relative frequency of phage type 42 D/1 in various materials

The incidence of phage type 42 D/1 in nasal specimens of infants varied considerably during the 41 week observation period. Fig. 3 shows the data in comparison with the total number of staphylococci isolated from nasal secretions.

Of the peaks and declines in Fig. 3 the minima observed on the 13th, 20—22nd, 29th and 36th weeks deserve some interest. The fall in the incidence of carriers might have been associated with the administration of new prophylactics (hexachlorophen dusting powder and nasal ointment and neomycin nasal ointment). The fact that the results obtained with these drugs were always better at the beginning than later, may be attributed to a more careful work of the staff at the time of the introduction of the new drug. The rises and falls in the curve may be associated with variations in the incidence of 42 D/1 carriers among the staff. At the beginning of the outbreak, 5 out of 10 nurses working in the infants' ward harboured phage type 42 D/1 staphylococci. Substitution of the "dangerous" nurses, because of staff shortage, could be

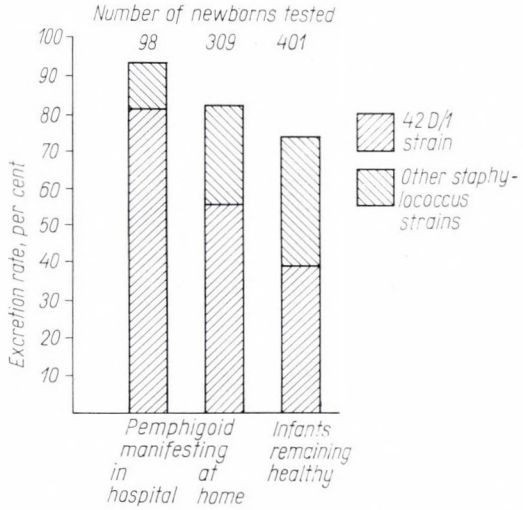

Fig. 2. Incidence of staphylococcal carriers among infants with and without pemphigoid

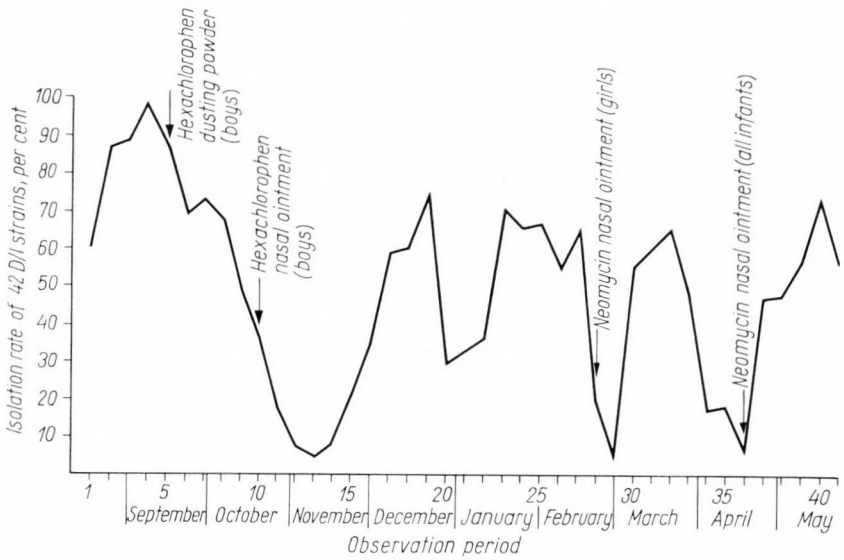

Fig. 3. Incidence of phage type 42 D/1 in infants during stay in hospital

performed only gradually in 10 weeks. The curve shows a parallel decrease in the number of 42 D/1 carriers among the infants. The prophylactic measures were insufficient either to eradicate the organism or considerably to decrease its incidence; the curve rose again from the 15th week onward, and showed some other peaks which, however, were lower than the original maximum.

The invasiveness of the causative agent is well demonstrated in Fig. 2, where it is seen that pemphigoid was more common among nasal carriers than

Table III

Antibiotic resistance of 2810 Staph. aureus strains according to phage groups

Phage group	No. of strains	Incidence of antibiotic resistant strains, per cent					
		Penicillin	Strepto-mycin	Chlor-amphenicol	Tetra-cycline	Neomycin	Erythro-mycin
I	261	70.5	9.2	4.9	1.9	2.6	13.0
II	114	77.2	9.6	43.0	1.7	2.6	5.2
III	271	90.9	54.2	49.4	51.6	2.5	48.7
M	40	15.0	10.0	2.5	0.0	5.0	2.5
Mixed	109	86.2	47.0	4.6	17.4	24.7	37.6
42 D/1	1238	99.8	87.9	12.6	21.3	3.4	97.8
Other additional .	78	84.3	14.0	5.1	11.5	1.2	9.0
Untypable	699	75.1	51.5	9.3	10.0	0.3	58.5
Total	2810	86.9	58.1	13.4	18.1	3.2	65.0

among infants not harbouring the organism. Similar data were obtained for infants discharged from hospital without symptoms. Of 541 such infants 232 were nasal carriers of 42 D/1, and 150 of other staphylococci; the remaining 159 infants were bacteriologically negative. In the first group 61.2, in the second 38.0, and in the third 28.3 per cent, subsequent cases of pemphigoid occurred. The incidence of pemphigoid appearing in the hospital and at home as compared to the incidence of nasal carriers is demonstrated in Fig. 4.

The abscissa in Fig. 4 represents the examination period divided into 4 parts: in part 1 no antistaphylococcal measures were applied; in parts 2, 3 and 4 different prophylactic drugs (hexachlorophen dusting powder, hexachlorophen nasal ointment and neomycin nasal ointment) were administered. The curves indicate percentage values for the incidence of all staphylococcal carriers, 42 D/1 carriers and pemphigoid infections among newborn infants. It is evident that the incidence of carriers gradually decreased especially after

Table IV

Incidence of antibiotic resistant 42 D/1 and untypable staphylococcal strains in carriers

Source of strains	Phage type	No. of strains	Antibiotic resistant strains, per cent					
			Penicillin	Strepto-mycin	Chlor-amphe-nicol	Tetra-cycline	Neomycin	Erythro-mycin
Nasal samples, infants	42 D/1	647	100.0	90.6	11.4	23.8	2.6	99.7
	Untypable	283	90.5	67.5	11.3	12.8	0.4	78.8
Nasal samples, mothers	42 D/1	123	99.2	89.3	7.4	13.9	8.2	83.6
	Untypable	181	40.3	18.8	3.4	5.6	0.6	16.6

the application of prophylactic agents. In parts 1 to 3 the relative number of 42 D/1 carriers also decreased, probably owing to the continuous substitution of the Staphylococcus-positive staff. The number of pemphigoid cases manifesting itself in and outside the hospital also decreased within these periods. In the last period, however, the number of cases increased parallel with the relative increase in the number of 42 D/1 carriers, despite the fact that the absolute number of staphylococcal carriers was the lowest during part 4.

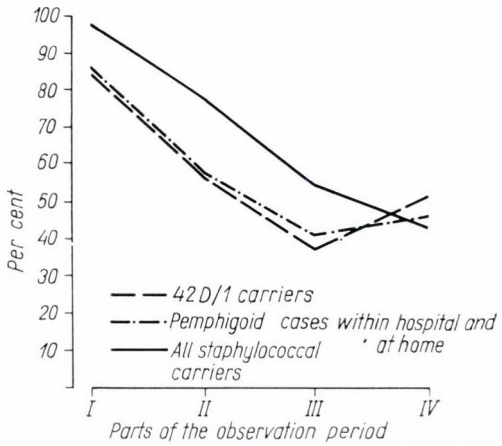

Fig. 4. Incidence of staphylococcal carriers and pemphigoid cases in four subsequent parts of the observation period

In this period the number of infective sources had probably increased in the newborn ward. It should be noted again that throughout the described epidemic, infants harbouring phage type 42 D/1 were more liable to develop pemphigoid than those negative for this organism.

The frequency of staphylococcal phage types resistant to various antibiotics is shown in Table III. The highest number of antibiotic resistant cultures occurred among phage group III, type 42 D/1 and untypable strains. Phage group III occurred infrequently; the majority of the cultures belonged to type 42 D/1 or were untypable. This explains the unusually high frequency of erythromycin resistant cultures; phage type 42 D/1 was resistant to this antibiotic in 97.8 per cent. The comparison of the antibiotic resistance of nasal 42 D/1 and untypable strains isolated from mothers and infants is interesting (Table IV).

It is seen in Table IV that the antibiotic resistance of phage type 42 D/1 strains was independent from the source. In contrast, untypable strains isolated from mothers were more frequently sensitive than those isolated from infants. It is justified to assume that the mothers acquired 42 D/1 strains after, while most of the other staphylococci before, admission to hospital.

Discussion

In recent years the pathogenic role and high spreading capacity of staphylococci untypable with the generally used set of phages, have been described by several authors. Thus in England JACOBS *et al.* [5], TURNER and WILLIS [6], TEMPLE and BLACKBURN [7], MITCHELL [8], in Canada COMTOIS and BYNOE [9] have described the occurrence of nosocomial strains lysed by group III concentrated phages but not by phages used in the routine test dilution. These highly virulent new epidemic staphylococci produced abundant yellow pigment and were generally resistant to penicillin and tetracyclines. Twenty-five per cent of staphylococci isolated by SOMPOLINSKY [10] in Israel reacted only with a new phage. Similarly, our phage type 42 D/1 strains can be regarded as new epidemic staphylococci.

The mere prevalence of a certain staphylococcal phage type in a hospital ward does not necessarily indicate its infectivity and responsibility for the outbreak. WILLIAMS and JEVONS [11] and other authors suggest that various staphylococci differ in infectivity and therefore not all strains of this ubiquitous organism are able to cause epidemics. This conception has not yet been confirmed experimentally; although observations indicate differences *in vivo* we lack the method for the detection of such strains *in vitro*.

Nosocomial infections are frequently due to phage type 80/81 or related lysis pattern strains. These presumably highly virulent strains have extensively been studied, yet, no characteristic properties differentiating them from other staphylococci have been demonstrated. ROUNTREE [12], who was the first to describe these strains, assumed that their virulence was associated with their capability to penetrate through the intact skin. This conception has not yet been confirmed. In pyogenic lesions other staphylococci belonging to phage group I are encountered frequently [13—17]. From nasal samples of patients and staff phage group III strains were isolated [18, 19]. Multiple antibiotic resistance, which is characteristic of nosocomial strains, occurred mainly among group III staphylococci. Yet, as it has been shown also by MILCH *et al.* [20], group III staphylococci are less frequently responsible for epidemics in Hungary than group I strains. To our best knowledge, in this country there has been no example of a such widespread outbreak as that described in the present paper. Even where a great communicability of dangerous types has been experienced, not more than 20 to 25 per cent of the exposed patients became affected [21].

It is not known why phage type 42 D/1 was prevailing during the whole observation period. The fact that this phage type was most common among newborn infants at the beginning of the outbreak explains the findings only partly, since on the basis of the observed frequency, this organism could not overpower other staphylococci. For the latter there would have been also an

ample chance during the 8 month period to give rise to mass outbreaks of pemphigoid. The number of infections due to phage type 42 D/1 showed periodical variations. Staphylococcal infections were generally caused by this phage type even in periods when its relative incidence was not too high. It seems therefore justified to conclude that phage type 42 D/1 must be regarded as dangerous organism of special virulence.

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THE OCCURRENCE OF MICROSPORON COOKEI IN HUNGARY

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(Received November 14, 1964)

Summary. A dermatophyte fungal species, *Microsporon cookei* Ajello 1959 has been isolated from Hungarian soil.

Microsporon cookei described by COOKE was at first regarded as a red pigment-producing variant of *Microsporon gypseum* [1]. A more detailed study of the organism was performed by AJELLO in 1959 [2]. *M. cookei* occurs in soil and on the fur of various mammals. BLASCHKE-HELMESSEN isolated 24 strains from 140 soil samples [3]. Animals carrying the organism showed no pathological symptoms. It was non-pathogenic to laboratory animals. On one occasion it was isolated from the diseased skin of a dog; however, microscopic examination of scrapings from the lesion revealed no fungal elements. Still, it cannot be excluded that under certain circumstances the species is responsible for pathological conditions [2]. In 1962, 3 pigment fractions were isolated from *M. cookei*, which were identical with the pigments of *Trichophyton rubrum* [4]. Recently, KABEN [5], who succeeded in isolating the fungus from 4 soil samples has tabulated the results of other investigators as to the occurrence of the organism in soil. On the basis of worldwide surveys, it can be stated that among microbiologically dermatophytic species, in addition to *Trichophyton terrestre*, *Keratinomyces ajelloi* and *Microsporon gypseum*, only *M. cookei* occurs in soil as a saprophyte.

By means of the To-Ka-Va method [6, 7] in 1963 we isolated from 170 soil samples 58 *K. ajelloi*, 29 *M. gypseum* and 11 *T. terrestre* strains [8]. In these examinations we found no *M. cookei* strains.

Results and conclusion

In the present studies nearly 100 soil samples were examined by the To-Ka-Va method [6, 7]. One of the samples originating from a road at Budaörs, yielded *M. cookei* together with *M. gypseum*. *M. cookei* differed from the latter organism in the presence of a reddish-violet pigmentation. The strain was isolated on Sabouraud glucose agar. Microscopic examination revealed

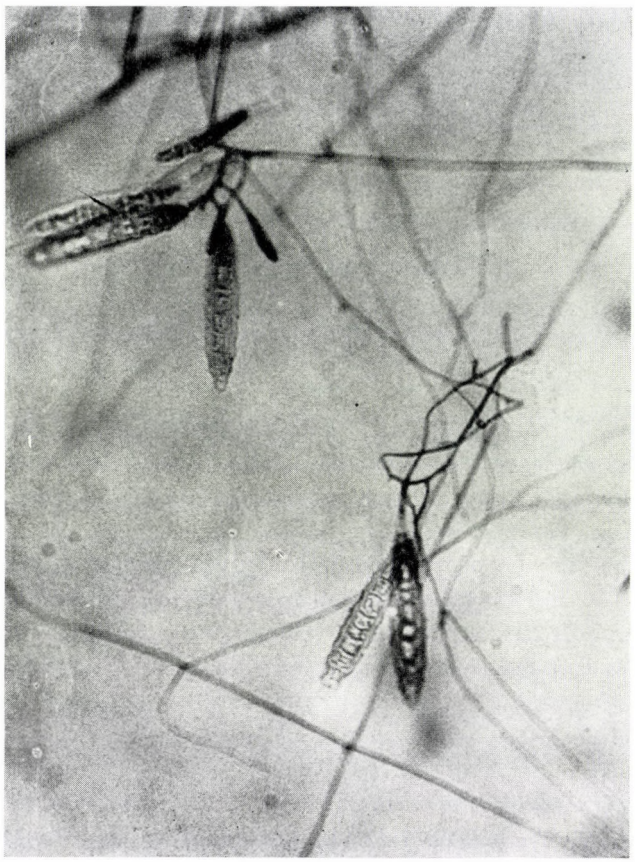

Fig. 1. Microculture of *M. cookei*. Schiff's periodic acid staining. Magnification, approx. × 500

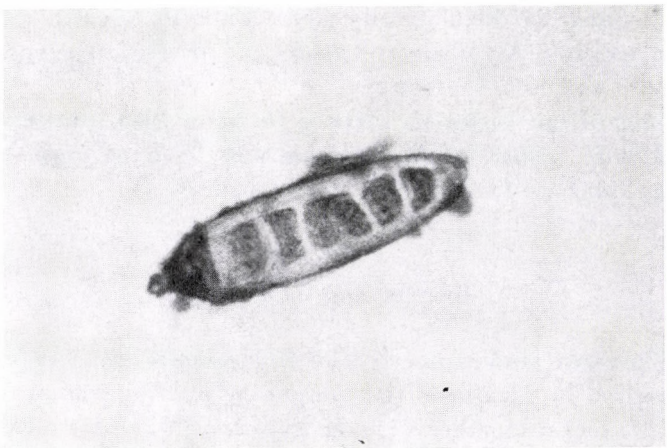

Fig. 2. Thick-walled macroconidium of *M. cookei*. Lactophenol-cotton blue staining. Magnification, approx. × 1000

rough and very thick macroconidial walls. This property in addition to pigment production, is the most characteristic feature of *M. cookei*. The strain was identified on the basis of data described in the literature and of a comparison with *M. cookei* type strains of our collection [3].

In the present examinations in addition to *M. gypseum*, *K. ajelloi* [9,8] and *T. terrestre* [8] the occurrence in Hungarian soil of the fourth ubiquitous dermatophytic species, *M. cookei* has been revealed.

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EFFECT OF HEPARIN ON THE GROWTH OF THE HERPES GROUP OF VIRUSES

By

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(Received November 28, 1964)

Summary. The effect of heparin was studied on the growth of Herpesvirus varicellae, Cytomegalo- and Herpes simplex viruses in secondary human fibroblast, HeLa and secondary human thyroid tissue cultures. Heparin was added to the cultures at different points of time following infection. The titre (Herpes simplex virus) resp. focus count reduction (Varicella and Cytomegalovirus) was the highest in cultures to which virus and heparin had been added simultaneously. The grade of inhibition depended on the actual concentration of heparin and the type of tissue culture used. In the presence of 10 μg/ml of heparin, the infective titre of Herpes simplex virus was reduced by 1.5 and 3.5 logs in secondary human fibroblast and in HeLa cell cultures, respectively. Experiments with intracellular Cytomegalovirus have shown that heparin inhibits only the adsorption of free virus and does not influence the penetration and cell-to-cell transfer of viruses carried by infected cells.

Several reports have been published on the effects on virus replication of different biological and synthetic substances. One group of these substances comprises anion polymers. Among them, polyfluoroglucinol phosphate and dextrane sulphate are known to inhibit the growth of Herpesvirus. Heparin is a similar polymer, consisting of mucoitin polysulphonic acid containing 40 per cent of sulphuric acid esterified with acetylglucosamine and glucuronic acid.

The effect of heparin on virus reproduction was first studied with Herpes simplex virus by VERDI and SERRA in 1956 [1]. The viruses of encephalomyocarditis [2] and poliomyelitis [3] were also examined in this respect. Recently, VAHERI and CANTEL [4] have performed studies on the interactions of heparin and viral growth.

In the present study the effect of heparin has been investigated on Herpesvirus varicellae, Cytomegalo- and Herpes simplex viruses. This attempt has been thought to be of interest, heparin being a physiological substance and the viruses chosen for study being capable of persisting in the organism in a latent form for prolonged periods.

Materials and methods

Tissue culture media. Primary cultures of human embryonic tissue were prepared in PARKER's 199 solution with 20 per cent calf serum. After 24 hours the medium was substituted with HANKS' solution containing 0.5 per cent lactalbumine hydrolysate and 10 per cent of calf serum. The latter medium was used for maintaining the culture until used. HeLa cells

were cultured in PARKER's medium containing 10 per cent of calf serum. As a maintenance solution the same medium, containing 5 per cent of calf serum was used. Thyroid cells were cultured in a mixture of 50 per cent PARKER's solution, 40 per cent HANKS' solution with 0.5 per cent lactalbumin hydrolysate content and 10 per cent of calf serum.

Tissue cultures. Human embryonic fibroblast cultures were obtained by trypsinization of 2 to 3 month-old embryos. Primary cultures were set up in Roux-flasks. Secondary cultures were prepared in tubes with cells obtained by trypsinization of the primary cultures. The secondary cultures were usually 2 to 3 days old when used. A similar method was employed for the preparation of human thyroid cell cultures using surgically removed thyroid glands. HeLa cells were cultured in 1 litre Roux flasks. Tube cultures were prepared from these stock cultures and used when 2 to 3 days old.

Viruses. Varicella virus was isolated from a typical vesicle and maintained in secondary human fibroblast cultures. In the system used, Varicella virus was mainly intracellular, thus when +++ cytopathogenic effect had developed, the cultures were trypsinized (0.15 per cent trypsin) and the cells made up in fresh medium to a final volume of 0.6 ml. This was taken as undiluted virus. Cytomegalovirus was obtained by the courtesy of Dr. H. STERN (St. George's Hospital Medical School, London), and maintained in our laboratory in secondary human fibroblast cell cultures. Infected cultures were used either as a whole or as cells and fluid phase separately. In some experiments the infected cells were disrupted by sonication (10 seconds, 1.7 Amp, MSE apparatus). Herpes simplex virus was isolated from the conjunctival washings of a patient with keratoconjunctivitis [5]. The strain was maintained in HeLa cell cultures.

Determination of infectivity and focus reduction. Viruses were titrated in 10fold dilution series inoculating 0.1 ml per dilution into 3 to 4 parallel tubes each. Readings were made daily during a period of 6 days. Results presented in this paper are those obtained on the 5th day. Infectivity titres were calculated according to KÄRBER. In focus reduction experiments a 50 per cent reduction of focus count was considered significant.

Heparin was obtained from the G. Richter Pharmaceutical Works, Budapest, and used at 0.1, 1 or 10 μg/ml.

Results

In the first series of experiments, heparin treatment was performed simultaneously with the infection. Parallel titrations of Herpes simplex virus, Herpesvirus varicellae and Cytomegalovirus were performed in the absence and in the presence of 0.1, 1 and 10 μg/ml of heparin. Herpesvirus varicellae was titrated in both secondary human fibroblast and thyroid cell cultures.

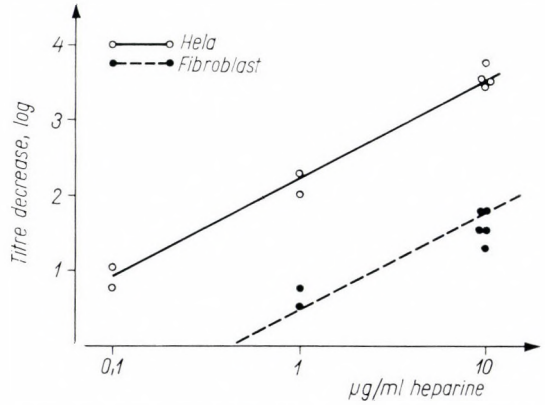

Fig. 1. Effect of heparin on the titres of Herpes simplex virus in HeLa and secondary human embryonic fibroblast cell cultures. Titre decreases as compared to heparin-free controls

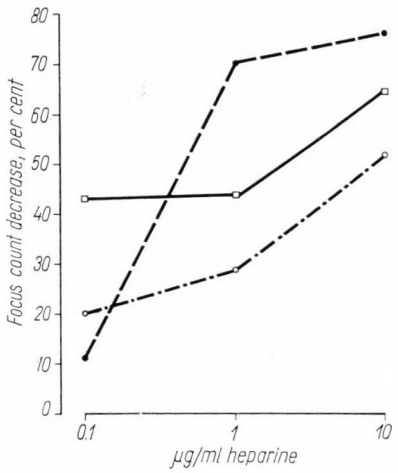

Fig. 2. Effect of heparin on the titre of Varicella and Cytomegaloviruses in human fibroblast and thyroid cell cultures. Reduction of focus count as compared to the appropriate controls.

●———● Varicella virus in human fibroblast cells
□———□ Cytomegalovirus in human fibroblast cells
○—·—·○ Varicella virus in human thyroid cells

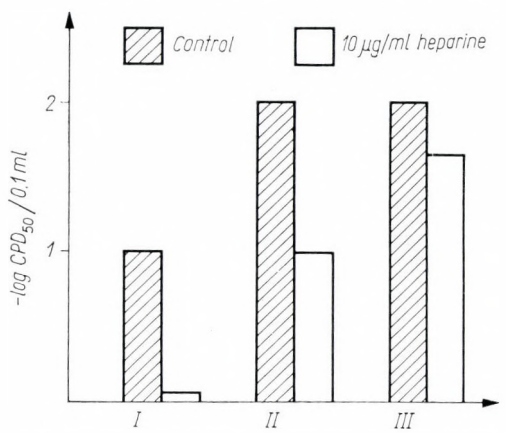

Fig. 3. Effect of heparin on the infectivity of Cytomegalovirus. Types of inocula: I. Infectious culture fluid (extracellular virus). II. Sonicated total infected culture. III. Virus-infected cell suspension (intracellular virus)

Herpes simplex virus was titrated in both secondary human fibroblast and HeLa cells while Cytomegalovirus was titrated in cultures of the former cells only. Results are presented in Figures 1 and 2.

A direct correlation was found between the concentration of the applied heparin and the titre decrease of Herpes simplex virus (Fig. 1). The lowering of titre was remarkably greater in HeLa than in human embryonic fibroblast cell cultures. With Varicella and Cytomegaloviruses even the highest dose of

heparin caused a titre decrease within the order of one log only. This lowering of focus count was, however, significant in all cases at every concentration of heparin (Fig. 2). Values presented in the graphs are calculated averages of 3 or 4 experiments. The effect of heparin on Varicella virus was somewhat more intensive in human embryonic fibroblast, than in human thyroid cell cultures.

The viruses used are known to differ in their tendency to appear extracellularly. The greater this tendency of a virus the more pronounced the inhibitory effect of heparin. Varicella and Cytomegaloviruses being localized mostly intracellularly, they exhibited titre decreases only within the order of one log in the heparin-treated cultures.

In the second series of experiments the effect of heparin on the growth of Cytomegalovirus was studied in cultures infected with extracellular (infectious culture fluid) or intracellular (washed suspensions of infected cells) virus. In some other experiments sonicated total infected cultures were used as an inoculum. The experiments were performed in secondary human embryonic fibroblast cells using a final heparin concentration of 10 μg/ml. Results obtained are given in Fig. 3. As compared to the heparinless controls there was only a slight difference demonstrable in the heparinized cultures inoculated with "intracellular" virus. A marked inhibition was, however, noted when the "extracellular" virus was used as inoculum. It appeared to be irrelevant whether the "extracellular" virus was an infective culture fluid or a sonicated total culture.

Experiments were performed with HeLa cell cultures pretreated with 10 μg/ml heparin for 24 hours prior to inoculation with Herpes simplex virus. The titre decrease was about 1 log in these experiments as compared to the remarkable lowering observed in cultures to which virus and heparin had been added simultaneously (Fig. 1).

A similarly moderate inhibition (1 log) occurred when heparin had been added one hour after the infection.

The direct effect of heparin on extracellular Herpes simplex and intracellular Varicella and Cytomegaloviruses was also studied. Culture fluid resp. suspensions of infected cells were mixed with heparin at 10 μg/ml final concentration and allowed to stand at room temperature for 3 hours. The mixtures were then titrated in HeLa (Herpes simplex) or human fibroblast (Varicella and Cytomegaloviruses) cell cultures. Significant titre decreases were not demonstrable.

Discussion

In our studies heparin had the greatest inhibitory action on virus multiplication when it was administered simultaneously with the infection. Pretreatment with heparin of the cell cultures did not influence remarkably their

ability to support viral growth. Treatment with heparin for 3 hours at room temperature did not reduce the infectivity of any of the tested viruses.

In the systems used in the present study, Varicella virus was present only intracellularly, Cytomegalovirus mainly intracellularly and Herpes simplex virus both intra- and extracellularly. The multiplication-inhibitory action of heparin was most marked on the extracellular Herpes virus, the others exhibited an infective titre-decrease of about 1 log only.

The results suggest that heparin as an active polyanion exerts its inhibitory action at the level of virus adsorption. There are data on the fixation and growth inhibitory action of heparin in tissue cultures [6—10]. More recent papers [2, 11—14] and the present results, however, suggest the importance of the formation of heparin-virus complexes with moderate if any capacity to infect cells.

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RHODOTORULA ZSOLTII N. SP. A NEW SPECIES OF YEASTS, AND SOME NOTES ON THE TAXONOMY OF THE GENUS RHODOTORULA

By

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(Received December 11, 1964)

Summary. A new species of Rhodotorula, named *Rh. zsoltii* n. sp. has been described. It produces red coloured carotenoid pigment, assimilates glucose, galactose (weakly) and sucrose (weakly), but not maltose, lactose, raffinose or nitrate and ethanol. The species splits arbutin and produces no starch-like compounds.

During the investigation of the human yeast flora, from the faeces of three different infants three identical Rhodotorula strains have been isolated which on identification proved to be representatives of a new species.

Materials and methods

From three infants under one year of age, two of them healthy and the third suffering from gastrointestinal illness, faeces were collected in sterile tubes and isolations were made on SABOURAUD's glucose agar containing penicillin and streptomycin [1].

Subcultures were made on malt agar. The isolated and on PAGANO—LEVIN—TREJO's TTC-agar [12] purified strains were identified by the method of LODDER and KREGER-VAN RIJ [8] as modified by ZSOLT and NOVÁK [15]. The taxonomical position is given by the use of NOVÁK and ZSOLT's yeast-system [10] and HASEGAWA's taxonomical work on Rhodotorulas [2—6].

Results

I. Taxonomically valuable characters. (1) Sexual reproduction: lacking; (2) Vegetative reproduction: only by budding; (3) Assimilation of carbohydrates: D(G)(S)/MLR*; (4) Fermentation of carbohydrates: —/dgsmlr*; (5) Assimilation of nitrogen sources: $(NH_4)_2SO_4$ and peptone/KNO_3^*; (6) Assimilation of ethanol: negative; (7) Splitting of arbutin: positive or weakly positive; (8) Production of starch-like compounds: negative; (9) Production of carotenoid pigment: positive.

* The letters before the oblique fraction line represent the substances with positive result, while those after the line the negative ones. D and d = glucose, G and g = galactose, S and s = sucrose, M and m = maltose, L and l = lactose, R and r = raffinose. Capital letter symbolises the respective sugar in assimilation while the little one in fermentation. Nitrogen sources are designated by the usual chemical symbols. Symbols in parentheses represent weak or latent characters.

Table I

Species of the genus Rhodotorula

Subgenus Rubrotorula	D	G	S	M	L	R	NO_3	E	A	C	St	
1. *Rh. pallida* LODDER	+	+	−	−	−	+	−	−	+	p	−	
2. *Rh. zsoltii nov. spec.*	+	(+)	(+)	−	−	−	−	−	(+)	p	−	
3. *Rh. graminis* di MENNA	+	+	+	−	−	/	+	/	/	p,r	−	
4. *Rh. laryngis* REIERSÖL	+	+	+	+	−	−	−	−	/	r	−	
5. *Rh. slooffii* NOVÁK et VÖRÖS-FELKAI	+	+	+	−	+	−	−	−	+	p	−	
6. *Rh. minuta* (SAITO) HARRISON	+	+	+	−	−	+	−	+	+	o	−	Rh. minuta species group
7. *Rh. pilimanae* HEDRICK et BURKE	+	+	+	−	−	+	−	+	/	p, o	−	
8. *Rh. rubra* (DEMME) LODDER emend. HASEGAWA	+	+	+	+	−	1/3	−	(∓)	+	p, o	−	
9. *Rh. glutinis* HARRISON emend. HASEGAWA	+	+	+	+	−	1/3	(+)	±	+	r, o	−	
10. *Rh. texensis* PHAFF, MRAK et WILLIAMS	+	+	+	−	+	+	−	+	+	o	−	
11. *Rh. marina* PHAFF, MRAK et WILLIAMS	+	+	+	+	(+)	+	−	−	+	o	−	
12. *Rh. nitens* MACKENZIE et AURET	+	+	+	+	+	3/3	−	−	+	o	(+)	
13. *Rh. lactosa* HASEGAWA	+	+	+	+	+	+	+	+	+	o, p	−	Rh. infirmo-miniata species group
14. *Rh. infirmominiata* (OKONUKI) HASEGAWA et BANNO	+	+	+	+	+	+	+	−	+	o	+	

Table I (cont.)

Subgenus Flavotorula	D	G	S	M	L	R	NO_3	E	A	C	St
1. *Rh. luteola* (SAITO) HASEGAWA, BANNO et YAMAUCHI	+	+	+	+	−	/	−	+	+	y	+
2. *Rh. flava* (SAITO) LODDER	+	+	+	+	+	−	−	−	+	y, o	−
3. *Rh. gelatinosa* (SAITO) HASEGAWA, BANNO et YAMAUCHI	+	+	+	+	+	−	+	±	+	y, o	+
4. *Rh. diffluens* (ZACH) HASEGAWA, BANNO et YAMAUCHI	+	+	+	+	−	+	+	(±)	+	y, o	+
5. *Rh. laurentii* (KUFFERATH) HASEGAWA, BANNO et YAMAUCHI	+	+	+	+	+	+	−	±	+	y, o	+
6. *Rh. albida* (SAITO) nov. comb.	+	+	+	+	+	+	+	+	+	ly	+
Species questionably belonging to the subgenus Flavotorula											
1. *Cr. skinneri* PHAFF et CARMO-SOUSA	+	(+)	−	−	−	−	−	−	/	bu	+
2. *Cr. gastricus* REIERSÖL et DI MENNA	+	+	−	+	−	/	−	−	−	yb	+
3. *Cr. terreus* DI MENNA	+	+	−	+	+	/	+	−	+	yb	+
4. *Cr. terricolus* PEDERSEN	+	+	+	+	+	/	+	+	−	ly	(+)

D = glucose, G = galactose, S = sucrose, M = maltose, L = lactose, R = raffinose, $NO_3 = KNO_3$, E = ethanol, A = arbutin, C = carotenoid pigment, St = starch. + = positive, − = negative, (+) = weakly positive, (‡) = positive or weakly positive, (±) = positive or weakly positive or negative, ± = positive or negative, (∓) = weakly positive or negative, / = no data. Colour of the carotenoid pigment: p = pink, o = orange, r = red, y = yellow, ly = light yellow, bu = buff, yb = yellowish brown.

II. Accessory characters. (1) Growth in malt extract. 3 days, 25° C: cells are globose or ovoid, 3.8—5.0 by 5.0—7.5 μ, single or in pairs, rarely in triplets. Only sediment is formed. 30 days, 17° C: only sediment is seen. (2) Growth on malt agar. 3 days, 25° C: cells are ovoid, 2.5—3.8 by 5.0—6.3 μ, single or in pairs. 30 days, 17° C: colony is shiny, mucous and fluidal, and pink in colour. (3) Growth on potato glucose agar (slide culture): only budding cells are seen. (4) Growth on corn-meal agar slant [9]: only budding cells are seen. (5) Origin of strains: No. XLV/1964 (type strain) and No. XLVI/1964 from faeces of healthy infants, while No. XLVII/1964 from faeces of an infant suffering from gastrointestinal disease.

Discussion

As our strains produce a carotenoid type red pigment but no ascospores or ballistospores and no pseudo or true mycelium they fit into the genus Rhodotorula HARRISON emend. HASEGAWA et BANNO [4]. In the genus the new species has its position in the subgenus Rubrotorula — according to the red colour of the produced pigment — between *Rhodotorula pallida* LODDER and *Rh. laryngis* REIERSÖL [13], as seen in Table I.

The new species has been named *Rhodotorula zsoltii* n. sp. in honorem Dr. J. ZSOLT, an excellent cultivator of taxonomy and physiology of yeasts in Hungary.

In Table I a new system of Rhodotorulae is proposed, based upon the taxonomical work of LODDER and KREGER-VAN RIJ [8], NOVÁK and ZSOLT [10], and HASEGAWA *et al.* [2—6]. According to NOVÁK and ZSOLT [11], two species groups may be formed in the subgenus Rubrotorula; the first for *Rh. minuta* (SAITO) HARRISON and *Rh. pilimanae* HEDRICK et BURKE [7], and the second for *Rh. lactosa* HASEGAWA [2] and *Rh. infirmominiata* (OKONUKI) HASEGAWA et BANNO [3], as these species-pairs have the same combinations of the main taxonomical characteristics. We cannot agree with HASEGAWA and BANNO [4] in treating *Rh. minuta* as a variety of *Rh. texensis* and *Rh. pilimanae* as a synonym of *Rhodotorula rubra*, since *Rhodotorula minuta* is a lactose-negative species and therefore the strains showing a weak assimilation of lactose should be treated as *Rhodotorula texensis*. The superficial similarity of these two species does not suffice for drawing conclusions and until new examinations have solved this problem we shall treat them as separate species. As to the second question, we could not find any explanation in HASEGAWA's text [4] that would account for the synonymization of *Rh. pilimanae* (described by HEDRICK and BURKE as a maltose-negative species) and *Rh. rubra* (which species is maltose-positive). Other corrections made on HASEGAWA's system [4—6] are: (i) the admittance of *Rh. laryngis* REIERSÖL [13] into the subgenus Rubro-

torula; and (ii) the division of *Rh. albida* and *Rh. gelatinosa*. In connection with the latter we have to mention that we could isolate a strain (No. 34/1959) which was raffinose negative and fit into the description of *Rh. gelatinosa*, because we could demonstrate raffinose positivity with the type strain of *Cryptococcus albidus* (now *Rhodotorula albida*), similarly to several other strains of this species (XXXI/1962/V23—R, XXXI/1962/V30—TR, XXXI/1962/VI30—TD from air, and 373/1959, 316/1960, 153/1961, 85/1962, 461/1961, 481/1961—III from patients). These results have led to the separation of *Rhodotorula albida* (SAITO) nov. comb. from *Rh. gelatinosa* (SAITO) HASEGAWA, BANNO et YAMAUCHI.

Latin diagnosis

Ascospora, pseudomycelium et mycelium verum non formatur. Fermentatio nulla. In medio minerali cum glucoso, galactoso (exigue) et saccharo (exigue) sed non cum maltoso, lactoso et raffinoso crescit. Nitras kalicus non assimilatur. In medio cum alcohole aethylico non crescit. Arbutinum finditur. Amylum non componitur. In musto maltato (post tertiem diem, 26° C) cellulae gemmantes globosae vel subovoideae (3.8—5.0 × 5.0—7.5 μ), singulae aut binae. Sedimentum solum formatur. In agaro maltato (post tertiem diem, 26° C) cellulae gemmantes ovoideae (2.5—3.8 × 5.0—6.3 μ), singulae aut binae. Colonia (post unum mensem, 17° C) glabra, lucida et mucosa vel fluida, pallida rubra (cellulae pigmenta carotinoidea habent).

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ANTISTREPTOKINASE TITRE IN HEALTHY ADULTS

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Summary. The antistreptokinase titre in 400 healthy blood donors was examined with the author's modified method. A gamma-globulin preparation equivalent with the antistreptolysin-O standard was employed for comparison. The distribution of titres followed the rules of normal distribution in 96.25 per cent; the titres were lower than expected in 3.75 per cent. The average titre of the examined sera was 115.6 units. Titres exceeding 286 units were considered pathological. With the increase of the age of the examined persons the mean titres decreased. The ASK average for the total material as well as the ASK average for different age groups showed a good agreement with the average antistreptolysin-O titre determined in other blood donors.

We have developed a simplified method for the determination of antistreptokinase (ASK) titre [1, 2]. As no international standard has so far been established for the ASK unit, it was attempted to standardize the method. On the basis of certain considerations, the gamma-globulin preparation used as the Hungarian antistreptolysin-O (ASO) substandard was regarded as containing equivalent amounts of ASK and ASO units. It has been assumed that the population acquires a similar degree of immunity to various streptococcal exo-enzymes. Therefore, when compared to a standard containing equivalent amounts of the two antibodies, the ASO and ASK titres in healthy individuals should be similar in order of magnitude. To confirm this hypothesis, we have determined the ASK titre in 400 healthy blood donors.

It seemed desirable to investigate normal titres because only few results obtained with other methods in other countries have been reported in the literature [3—5].

Materials and methods

Serum samples were taken from Budapest residents aged 20 to 60 years, who at previous medical examination had been declared fit to supply blood for the transfusion service.

Titration. Inactivated sera were titrated by our modified method [1, 2] as follows. To 1.0 ml serum dilutions 0.5 ml ASK I, then after 15 minute incubation 0.5 ml ASK II reagent was added. After a subsequent 45 minute incubation the highest serum dilution inhibiting fibrinolysis was determined. The titre was calculated and expressed in units by comparing the corresponding reactions in the standard and examined serum dilution series. Lyophilized ASK I, ASK II and ASK standard preparations were produced in this institute.

Calculation. In our titration method the consecutive dilutions form a geometrical progression characterized by the quotient $\sqrt[4]{2}$. In the calculation of averages, limits of error, etc. titre grade numbers could therefore be substituted for absolute levels, and only the final results were expressed in titres by use of the formula shown in the note under Table I [6, 7].

Table I

ASK titre in 400 healthy adults

Titre	Titre grade	Number of subjects								
		Males				Females				Total
		20–30	31–40	41–60	Total males	20–30	31–40	41–60	Total females	
		years of age				years of age				
3.75	0	—	—	—	—	—	—	—	—	—
4.37	1	1	—	—	1	1	—	1	2	3
5.25	2	—	—	—	—	—	—	—	—	—
6.25	3	—	—	—	—	—	1	—	1	1
7.5	4	—	1	—	1	—	1	—	1	2
8.75	5	—	—	1	1	—	—	1	1	2
10.5	6	—	—	1	1	—	—	—	—	1
12.5	7	—	—	1	1	—	—	—	—	1
15	8	—	—	—	—	1	—	—	1	1
17.5	9	—	—	—	—	—	3	1	4	4
21	10	—	1	1	2	3	—	2	5	7
25	11	2	2	—	4	—	2	1	3	7
30	12	2	—	1	3	2	—	3	5	8
35	13	—	—	1	1	3	1	—	4	5
42	14	1	—	—	1	—	—	5	5	6
50	15	2	5	3	10	3	6	7	16	26
60	16	5	2	—	7	3	3	6	12	19
70	17	3	6	2	11	2	4	3	9	20
84	18	2	4	2	8	2	4	2	8	16
100	19	8	14	6	28	5	7	5	17	45
120	20	7	4	—	11	4	4	2	10	21
143	21	11	10	4	25	8	9	8	25	50
170	22	7	6	1	14	4	2	—	6	20
200	23	16	8	6	30	10	3	3	16	46
240	24	11	4	2	17	1	2	1	4	21
286	25	14	5	3	22	6	3	—	9	31
340	26	4	—	—	4	1	—	1	2	6
400	27	4	5	1	10	5	1	1	7	17
480	28	2	2	—	4	1	—	—	1	5
580	29	1	—	—	1	—	2	2	3	4
680	30	1	1	—	2	1	1	1	3	5
800	31	—	—	—	—	—	—	—	—	—

Average: $\bar{x} = \frac{zx}{n} = 19.79$ titre grades $= 115.6$ units

Standard deviation: $s = \sqrt{\frac{Sxx}{n-1}} = \pm 5.15$ titre grades

Deviation range: $\bar{x} \pm s = 14.64 - 24.94$ titre grades $= 47.4 - 282.3$ units

Note. Titre in units is calculated from any titre grade (Xn) with formula: $\left(\sqrt[4]{2}\right)^{Xn} \times 3.75$

Results

Age and sex distribution of ASK titres in sera of 400 healthy blood donors is presented in Table I. The average titre was 115.6 units, with a deviation range of 47 to 282 units. The most frequently occurring values were actually between 50 and 286 units, thus within the calculated deviation range. The frequency distribution followed the Gaussian curve only partly, as some of the results fell into very low titre ranges. The observed distribution was therefore compared with the normal distribution. A precondition of such

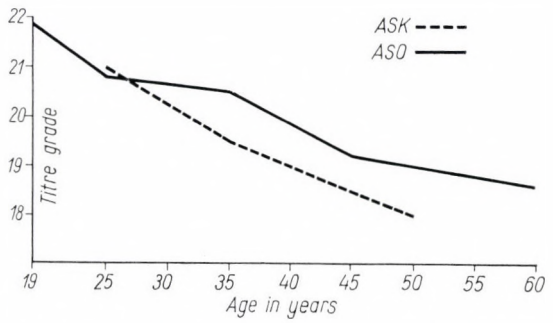

Fig. 1. Normal ASO and ASK levels in different age groups

calculations is that a certain percentage of titres should fall between the calculated average and the 1, 2 or 2½-fold limits of the calculated standard deviation [8]. The adequacy of our data to the above requirement is shown in Table II. It is seen that 96.25 per cent of the results corresponded to the normal distribution and only 3.75 per cent of the sera gave a titre lower than expected. This finding will be dealt with when discussing the results.

Data of Table I divided into low, medium and high titre groups are presented in Table III. Thus 78.75 per cent of the sera yielded titres ranging from 50 to 286 units. The separately calculated average for this medium titre group was 127 units. The remaining 21.25 per cent of sera fell into the low and high titre groups. The deviation extended over 5 titre grades upwards and 14

Table II

Frequency distribution of titres in various deviation ranges

Deviation range	Titre grade	Required	Observed
		frequency, per cent	
$\bar{x} \pm 1\ s$	14.64—24.94	68.27	71.00
$\bar{x} \pm 2\ s$	9.49—30.09	95.45	96.25
$\bar{x} \pm 2.5\ s$	6.92—32.66	99.00	97.75

Table III

Distribution of ASK levels according to titre groups

Titre group	Titre	Titre grade	No. of sera	Per cent
Low	4— 42	1—14	48	12.0
Medium.....	50—286	15—25	315	78.75
High	340—680	26—30	37	9.25
Total	4—680	1—30	400	100.0

titre grades downwards. The absolute number of sera in the low titre group was also larger than the number of sera in the high titre group (48 vs 37).

Summarized data according to age and sex groups are presented in Table IV. It is seen that with the increase of age the average titres decreased considerably in both males and females. The average titre was approximately 30 per cent higher in males than females. We have no explanation for the sex

Table IV

Average ASK titres of different age and sex groups

Age group	Males	Females	Total
20—30	158	120	142
31—40	128	92	111
41—60	97	76	84
20—60	135	95	115

difference; it may have been due to a chance distribution obtained in a relatively small number of individuals. It should be noted that among 600 blood donors examined 3 years ago, the female group yielded higher ASO titre [9].

It is of interest to compare the average ASK titres and the above mentioned ASO titres according to age groups (Fig. 1). It is obvious that the ASK and ASO level curves obtained for different donors with a 3 year difference were similar in course and showed some divergence only in the small oldest age group.

Discussion

Examination of the ASK level in 400 blood donors revealed that 96.25 per cent of the titres corresponded to the Gaussian distribution curve. Very low titres showing a wide deviation from the most prevalent values were

obtained in the remaining 3.75 per cent. This finding is somewhat similar to that reported by the Commission on Acute Respiratory Diseases [4], namely that ASK was absent from the sera of 27 per cent of 236 healthy soldiers. This observation indicates that part of the normal adult population had acquired no immunity to the streptokinase antigen. Owing to technical factors, the low levels may have been even lower. Our experience is namely that levels lower than 20 units cannot always be exactly determined because of the effect of plasminogen and other factors in the necessarily low serum dilution (1 : 1.25). Determination of low titres is not of practical interest as only normal and pathologically elevated titres are of diagnostic importance. The average titre obtained in our material was 115 units. About 80 per cent of sera fell within the titre range of 50 to 286 units. The separately calculated mean for the latter group was 127 units. Therefore, 120 units are considered normal for the ASK titre in healthy adults. The upper level is at most 286 units; such and higher values are pathological with a probability of 90 per cent. The upper limit is in fair agreement with the 256 unit level calculated by CHRIST [5], who examined 174 persons in Germany. The average titre found in our experiments is also in good agreement with the 125.6 units ASO titre regarded as the normal average in a healthy population [9]. The agreement between ASO and ASK normal titres is valid not only for the total material, but also for different age groups. The agreement between the total and age group averages indicates that the population is evenly exposed to antigenic stimuli by different streptococcal exo-enzymes. Therefore it is not only advisable but also justified to express the antibody levels to different streptococcal antigens in equivalent titre grades and units.

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COMPARISON OF ANTISTREPTOKINASE AND ANTISTREPTOLYSIN-O TITRE IN HOSPITALIZED CHILDREN

By

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Summary. The ASO and ASK titres in 500 hospitalized children have been examined. It has been concluded that of the two reactions ASK is a somewhat more specific indicator of streptococcal infection. Parallel determination of the two titres is advisable as in 26 per cent of the cases streptococcal aetiology was indicated by one of the reactions only. ASK titres appeared to extend over a wider range than ASO titres. With the method used in this work, border values between normal and pathological titres fell in the range of 240—280 units for both reactions.

Since TODD's report in 1932 [1], antistreptolysin-O (ASO) reaction has been regarded an important diagnostic test for the serological estimation of streptococcal aetiology in certain diseases. Later it has been shown that this reaction is not always indicative of streptococcal history and, alternatively, it may give false positive results without previous streptococcal infection. Negative reactions may be due to a low streptolysin-O antigen content of some A group streptococci. Thus RANTZ *et al.* [2] found raised ASO titres only in 79.5 per cent of 196 patients whose streptococcal infection had been confirmed by cultivation and type determination. In contrast, a high false positive titre is obtained in sera contaminated with some bacteria, especially with pseudomonads [3—5]. Certain pathological conditions such as hepatitis [6] and the nephrotic syndrome [7] may also present elevated ASO titres. The serum of healthy individuals may contain the so-called OSL-inhibitor [8, 9]. In view of the above disadvantages of the ASO reaction, several authors emphasize that streptococcal antibodies should be examined with 2 or 3 different antigens [10—14]. Titration with even more antigens was also recommended; these reactions have not been accepted for routine work, owing to technical difficulties and the lack of suitable international standards.

A method for routine antistreptokinase (ASK) titration [15], standardization of its results [16] and preparation of reagents [17] has been described by one of us. The new method is technically more simple than the ASO reaction. The ASK unit has been standardized so that the obtained titres are similar in order to the internationally standardized ASO titres. It should be noted that our ASK unit closely approximates the unit chosen by CHRISTENSEN [18].

Materials and methods

Serum samples were taken from 500 hospitalized children aged 2 to 12 years. Of the children 257 suffered from streptococcal and 113 from non-streptococcal diseases. Streptococcal aetiology was doubtful in 130 cases. In the results only the titre of one sample was included for each child, independently of the duration of hospitalization.

ASO and ASK titrations were performed as described by BÖSZÖRMÉNYI [19, 15]. Reagents were prepared by the Institute for Serobacteriological Production and Research "Human" Budapest. In grouping and evaluation of titres, as in both methods geometrical serial dilutions have been employed, titre grades could be substituted for absolute values [20].

Bacteriological examination of throat and nose samples was carried out on blood agar plates.

Results

ASO and ASK titres were determined in parallel examinations in 500 hospitalized children. The patients were divided into 6 groups on the basis of clinical and bacteriological findings. Group "rheumatic fever" included mainly patients with rheumatic carditis and smaller numbers of children with acute polyarthritis. Cases of chorea minor and glomerulonephritis constituted the second and third group of streptococcal diseases. Other streptococcal infections (scarlet fever, erysipelas, bacteriologically positive tonsillitis, sinusitis, otitis media) comprised the next group. Group 5 contained patients with diseases of doubtful streptococcal aetiology (bronchitis, bronchopneumonia and other respiratory infections, osteomyelitis, erythema nodosum and tonsillitis or sore throat with negative bacteriological findings). The group in which streptococcal aetiology could be excluded contained chickenpox, measles, influenza, neuropathy, nephrolithiasis, alopecia areata, congenital heart defects, leukaemia, epilepsy, cystitis, Perthes' disease, agammaglobulinaemia, anaemia, hypacidity and pleurisy.

The obtained titres fell in the range of 21 to 6400 and were distributed among 34 different levels. For the sake of simplicity 7 categories each including 4 titre grades were set up. Thus the lowest titre in one category was 100 per cent lower than the highest titre in the same category. As in previous examinations [19, 21] the border between normal and pathological titres fell into the range of 240 to 286 units, according to the above grouping the first three categories included normal, the others pathological titres. Table I summarizes the results according to disease groups and titre categories. The average titres for each disease group are presented in Table II. It is seen that, with the exception of the group including relatively small numbers of glomerulonephritic patients, practically uniform ASO and ASK titres were obtained. The average titres in the four groups of streptococcal diseases were well within the pathological range. Where their streptococcal origin could be excluded, the average titre fell in the normal range. In comparing ASO and ASK titres, a minimum difference was generally found; the average ASK titre

Table I

Distribution of ASO and ASK titres according to clinical diagnosis

Titre grade	Titre	Rheumatic fever		Chorea minor		Glomerulo-nephritis		Other strepto-coccal diseases		Strepto-coccal aetiology doubtful		Non-strep-tococcal diseases		Total	
		ASO	ASK	ASO	ASK	ASO	ASK	ASO	ASK	ASO	ASK	ASO	ASK	ASO	ASK
9—13	21— 42	1	2	—	—	1	1	1	2	20	15	22	18	45	38
14—18	50— 100	6	6	—	1	2	3	3	5	12	13	28	20	51	48
19—23	120— 240	14	18	3	5	8	1	14	11	32	49	23	41	94	125
24—28	286— 560	58	43	9	7	4	7	24	23	48	33	27	24	170	137
29—33	672—1344	38	51	11	7	4	7	11	14	16	18	7	7	87	104
34—38	1600—3200	30	17	3	6	1	1	10	7	2	2	6	3	52	36
39—42	3740—6400	1	11	—	—	—	—	—	1	—	—	—	—	1	12
Total		148		26		20		63		130		113		500	

was, however, somewhat lower in doubtful cases and somewhat higher in streptococcal infections than the average ASO titre.

Table I shows the ASO and ASK titres separately. In estimating the value of the two reactions, it was necessary to compare the titres obtained with the two reactions in individual sera. Therefore the results have been grouped in square tables, where the columns represent ASK categories, and the rows ASO categories (Tables III, IV and V). Evaluation was further simplified by using percentage values for the distribution of cases and by combining streptococcal diseases (rheumatic fever, chorea, glomerulonephritis and other streptococcal

Table II

Distribution of average ASO and ASK titres according to clinical diagnosis

Diagnosis	No. of cases	ASO		ASK	
		Titre grade	Titre	Titre grade	Titre
Rheumatic fever	148	28.36	602	28.75	644
Chorea minor	26	28.57	624	28.80	650
Acute glomerulonephritis	20	23.90	278	25.25	351
Other streptococcal diseases	63	26.58	442	26.93	470
Doubtful streptococcal aetiology	130	22.36	213	22.15	205
Streptococcal aetiology excluded	113	20.52	155	20.60	157

Table III

Percentage distribution of ASO and ASK titres in streptococcal diseases
Total number of cases, 257

Titre grade → / Titre ↓			Antistreptokinase 9–13	14–18	19–23	24–28	29–33	34–38	39–43	Total %
			21–42	50–100	120–240	286–560	672–1344	1600–3200	3740–7480	
Antistreptolysin-O	9–13	21–42		0.77	0.38					1.15
	14–18	50–100	0.38	0.38	1.16	1.94	0.38			4.24
	19–23	120–240	0.77	1.55	2.33	6.61	3.11	0.77		15.14
	24–28	286–560	0.38	1.16	5.05	12.84	13.22	3.50	0.77	36.92
	29–33	672–1344	0.38	1.16	2.72	5.83	9.33	3.50	1.94	24.86
	34–38	1600–3200		0.38	1.94	3.89	4.66	4.28	1.94	17.09
	39–43	3740–7480		0.38						0.38
Total %			1.91	5.78	13.58	31.11	30.70	12.05	4.65	99.78

infections) into one group. Thus the whole material is represented by 3 groups: streptococcal, doubtful and non-streptococcal diseases. If the ASO and ASK titre had been the same in every serum, all cases would have fallen in squares situated along the line running from the left upper corner to the right lower corner of the tables. It is seen that the results were far from being so uniform: considerable numbers of sera occurred which contained low titre antibodies against one enzyme and high (occasionally very high) titre antibodies against the other and vice versa. The wide discrepancy between the two titres in a number of individual serum samples is a further proof of the necessity of parallel

Table IV

Percentage distribution of ASO and ASK titres in diseases of doubtful streptococcal aetiology
Total number of cases, 130

Titre grade → / Titre ↓			Antistreptokinase 9–13	14–18	19–23	24–28	29–33	34–38	Total %
			21–42	50–100	120–240	286–560	672–1344	1600–3200	
Antistreptolysin - O	9–13	21–42	4.61	3.84	6.15	0.76			15.36
	14–18	50–100	2.30		5.38	1.53			9.21
	19–23	120–240	3.07	0.76	9.23	7.69	3.07	0.76	24.58
	24–28	286–560	1.53	3.84	13.84	10.00	7.69		36.90
	29–33	672–1344			3.07	5.38	3.07	0.76	12.28
	34–38	1600–3200		1.53					1.53
Total %			11.51	9.97	37.67	25.36	13.83	1.52	99.86

determination. This is especially important when the level of one of the antibodies is normal and that of the other pathological. The percentage distribution of the examined material as to normal and pathological serum levels is presented in Tables VI, VII and VIII. The most important conclusion drawn from Tables VI and VIII is that in streptococcal infections there was an almost 80 per cent agreement between ASO and ASK positivity. In non-streptococcal diseases the streptococcal aetiology was excluded by the ASK reaction in 70 per cent, while by the ASO reaction in 65 per cent only. The incidence of cases yielding normal titres with one reaction and pathological titres with the other was 26 per cent in Table VI, 38 per cent in Table VII, and 28 per cent in Table VIII.

The results obtained with the two reactions in individual sera independently of the clinical diagnosis are grouped in Table IX. The data were calcu-

Table V

Percentage distribution of ASO and ASK titres in non-streptococcal diseases
Total number of cases, 113

Titre grade → / Titre ↓			Antistreptokinase 9–13 (21–42)	14–18 (50–100)	19–23 (120–240)	24–28 (286–560)	29–33 (672–1344)	34–38 (1600–3200)	Total %
Antistreptolysin-O	9–13	21–42	6.19	4.42	7.96	0.88			19.45
	14–18	50–100	3.53	5.30	12.38	3.53			24.74
	19–23	120–240	4.42	2.65	6.19	3.53	3.53		20.32
	24–28	286–560	1.76	4.42	7.96	7.07	2.65		23.86
	29–33	672–1344				5.30		0.88	6.18
	34–38	1600–3200		0.88	1.76	0.88		1.76	5.28
Total %			15.90	17.67	36.25	21.19	6.18	2.64	99.83

Table VI

Percentage distribution of pathological and normal ASO and ASK titres in streptococcal diseases
Total number of cases, 257

		ASK		
Titre		21–240	286–6400	Total
ASO	21– 240	7.72	12.81	20.53
	286–3740	13.55	65.70	79.26
Total		21.27	78.51	99.78

Table VII

Percentage distribution of pathological and normal ASO and ASK titres in diseases of doubtful streptococcal aetiology
Total number of cases, 130

		ASK		
	Titre	25—240	286—1600	Total
ASO	25— 240	35.34	13.81	49.15
	286—2284	23.81	26.90	50.71
	Total	59.15	40.71	99.86

lated by comparing ASK titre grades to ASO titre grades. The difference expressed in titre units is shown in Table IX. Differences not exceeding ±40 per cent (less than ½ tube difference) were neglected and the titres were regarded as uniform. When calculated in this manner, the antibody titres determined with the two reactions agreed in 29.2 per cent. In 76 per cent of

Table VIII

Percentage distribution of ASO and ASK titres in non-streptococcal diseases
Total number of cases, 113

		ASK		
	Titre	25—240	286—2688	Total
ASO	21— 240	53.04	11.47	64.51
	286—2284	16.78	18.54	35.32
	Total	69.82	30.01	99.83

sera the difference was less than ±340 per cent, thus, if for example one serum gave an ASO titre of 200, the ASK titre was not more than 670 or not less than 60. With the usual two-fold technique the difference was therefore within 2 dilution degrees, which is a good agreement and does not indicate more serious technical errors or a considerable difference in the antibody response to the two kinds of antigen. As to the remaining 24 per cent of sera the ASK titre was 4 to 19 times higher in 12.4 per cent and 4 to 45 times lower in 11.6 per cent than the ASO titre. In these cases the discrepancy was most probably due to different mechanisms, and only one of the two reactions furnished a reliable indication of the presence or absence of streptococcal aetiology.

Table IX

Distribution of ASK titres as compared to ASO titre = x

ASK titre	Per cent	
Between $9.6x$ and $19.2x$	1.2	12.4
Between $4x$ and $8x$	11.2	
Between $1.7x$ and $3.4x$	25.8	76.0
Between $1.4x$ and $\frac{x}{1.4}$ (identical)	29.2	
Between $\frac{x}{1.7}$ and $\frac{x}{3.4}$	21.0	
Between $\frac{x}{4}$ and $\frac{x}{8}$	8.4	11.6
Between $\frac{x}{9.6}$ and $\frac{x}{19.2}$	2.4	
Between $\frac{x}{22.4}$ and $\frac{x}{44.8}$	0.8	
Total	100.0	

Discussion

In the present study answers were sought to the following questions. (i) Is the modified ASK reaction suitable for revealing or excluding streptococcal aetiology? (ii) Is it necessary to perform ASK in addition to ASO titration?

As to question (i), the answer is definitely in the affirmative. In diseases of undoubtedly streptococcal origin the ASK, similarly to the ASO, was positive in 80 per cent. In non-streptococcal diseases these reactions were negative in 70 and 65 per cent, respectively. In patients grouped according to clinical diagnosis, the ASK reaction yielded generally low or very high titres, while the ASO reaction often gave non-characteristic, medium titres. As shown in Table I, slightly elevated titres in the range of 286 to 560 were obtained in 170 sera with ASO and only in 137 sera with ASK. This finding is in agreement with that of ANDERSON *et al.* [22] and CARRAZ *et al.* [11], who observed in scarlet fever and rheumatic fever significantly higher ASK than ASO levels. Accordingly, the ASK reaction is more valuable for diagnostic purposes.

As to question (ii), the number of sera in which one of the tests gave pathological and the other normal titres, should be considered. Such cases occurred in 26 and 28 per cent in streptococcal and non-streptococcal diseases, respectively (Tables VI and VIII). The corresponding percentage in the group containing doubtful cases was 38 (Table VII). Irrespective of the border values

between normal and pathological titres, the incidence of a more significant, 2 or more tube difference between the two reactions turned out to be 24 per cent (Table IX). If incongruous reactions are caused by technical factors (haemolysis, contamination, chemical impurity, false dilution), the result can be corrected after a repeated examination. When, however, incongruity is due to the nature of the aetiological agent or to some more durable property of the serum (weak antigen stimulus cholesterinaemia, OSL-inhibitor, hepatitis, nephrosis) repeated examinations do not yield better results. A striking difference between the two reactions may help the clinician to recognize disease, which otherwise might have been diagnosed falsely. When the two results coincide, the aetiological diagnosis is established with double certainty. Therefore, the parallel examination of both reactions is recommended.

Large numbers of examinations have shown that the technical procedure of the modified ASK reaction is simple and adequate for routine laboratory work.

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STUDIES ON STAPHYLOCOCCUS AUREUS L FORMS BLOCKED AT DIFFERENT STAGES OF CELL WALL SYNTHESIS

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Summary. (i) Four different L strains growing well in liquid medium have been obtained with penicillin treatment from *Staphylococcus aureus* strain 100.

(ii) In the presence of penicillin two of the L cultures produced about 5-fold the amount of acid-soluble acetyl amino sugar-containing nucleotides than the others.

(iii) The accumulated nucleotides were identified as uridine diphosphate-muramic acid peptides.

(iv) When penicillin was omitted from the medium, the Park nucleotide production by the stable L form remained at the same level, while production by the two L strains elaborating this substance at large amounts in penicillin-containing medium, decreased considerably.

Penicillin has been reported to inhibit formation of the mucopeptide structure in the cell wall and therefore to cause an accumulation of muramic acid uridine nucleotide precursors in the cell [1—5].

As to *Staphylococcus aureus*, in view of the failure to detect cell wall components, resistance and osmotic sensibility, it may be assumed that L forms of this organism are completely devoid of cell wall [6, 7].

As L forms are usually induced by treatment with penicillin or penicillin derivatives, it seemed interesting to investigate the stage of cell wall synthesis in which the antibiotic exerts its inhibitory action. In the experiments stable and unstable L forms of *Staph. aureus* strain 100 were used.

Materials and methods

Medium 1. Beef extract, 3.7 per cent; $Na_2HPO_4 \cdot 7H_2O$, 2.0 per cent; NaCl, 3.0 per cent; agar, 1.0 per cent; human serum, 10.0 per cent in distilled water; penicillin, 500 units/ml, pH 7.3.

Medium 2. Beef extract, 3.7 per cent; $Na_2HPO_4 \cdot 7H_2O$, 2.0 per cent; NaCl, 3.0 per cent; sucrose, 5.0 per cent; human serum, 10.0 per cent; in distilled water; penicillin, 500 units/ml; pH 7.3.

Bacterial strain. Staph. aureus 100.

Induction of L forms with penicillin was performed as described previously [8]. After streaking a 24 hour broth culture onto medium 1 and drying at room temperature for 1 hour, the plates were incubated at 37°C for 96 hours. From the plates showing L colonies blocks were cut and transferred into medium 2. The L forms produced an abundant granular deposit in this medium after 4 to 7 days. Further subcultivation was performed in medium 2.

Ultrasonic treatment was carried out in an ice bath for 1 minute by means of an MSE disintegrator operating at 1.7 amperes.

Determination of N-acetyl amino sugar-containing nucleotides was based on the method of STROMINGER [9]. The N-acetyl amino sugar ester was liberated by hydrolysis with 0.01 *N*

HCl at 100°C for 15 minutes and determined as described by REISSIG *et al.* [10]. A non-hydrolysed sample was used as a blank. As a standard preparation, N-acetyl-glucosamine (Serva) was used. The amount of N-acetyl amino sugar was expressed as N-acetyl-glucosamine.

Determination of NaCl was carried out as described by RUSZNYÁK [11].

Paper chromatography of N-acetyl amino nucleotides was performed by use of the solvent system recommended by ITO and SAITO [12]. After development the spots were cut out under ultraviolet illumination then extracted for 24 hours. Part of the extract was used for spectrophotometric nucleic acid determination. The other part was hydrolysed with 6*N* HCl for 24 hours at 105°C, dried *in vacuo* and used for chromatographic amino acid determination. Lysine, histidine, arginine, tyrosine and phenylalanine were detected at 22°C by the descending method, using Whatman's No. 1 paper and n-butanol: acetic acid : water (4 : 1 : 1 and 4 : 1 : 5) solvents. Aspartic acid, glutamic acid, serine, glycine, threonine and alanine were determined by descending chromatography on Schleicher—Schüll's 2043/b paper with phenol solvent saturated with pH 12.0 McFarren buffer. Proline, valine, methionine and leucines were detected by the descending method on Schleicher—Schüll's 2043/b paper; as a solvent, benzyl-alcohol saturated with pH 8.4 McFarren buffer : n-butanol (1 : 1) was used. A purified casein hydrolysate served as a control [13].

Results

In the present experiments 3 different L strains were derived from *Staph. aureus* strain 100 (L_1, L_2 and L_3). From the third subculture onward the L strains grew well in medium 2 in 48 hours at 37°C. For N-acetyl amino sugar ester determination 48 hour cultures were centrifuged at 7000 r.p.m. for 10 minutes, then washed in 3 per cent NaCl. This procedure did not harm the L forms and acetyl amino sugars were not released into the supernatant. After ultrasonic disintegration part of the suspension was used for dry material determination. The sample was dried to constant weight in an exsiccator over phosphorus pentoxide. The amount of NaCl regained at washing was determined by RUSZNYÁK's method and subtracted from the dry weight. The rest of the material was incubated in a 100° C water bath for 25 minutes then cooled and centrifuged. To the supernatant trichloracetic acid was added to give a final concentration of 5 per cent. The mixture was placed in an ice bath

Table I

N-acetyl amino sugar content of acid-soluble nucleotides in Staph. aureus L strains

No. of subculture	N-acetyl glucosamine, μg/mg dry weight**			
	L_1	L_2	L_3	LS
4	1.0	9.2	11.1	
6	1.1	9.5	12.5	Average, 2.0
8	Not continued*	10.0	11.3	
10		8.6	10.0	
15		11.2	10.6	

* Strain L_1 grew poorly in subculture 7 and was unable to multiply on further passages.
** Average of 2 parallel determinations.

for 60 minutes then centrifuged while cool. Trichloracetic acid was removed by shaking with ether five times.

After hydrolysing with 0.01 *N* HCl for 15 minutes at 100°C, N-acetyl amino sugars were determined. Data obtained for the non-hydrolysed controls were subtracted from the values. Determination of N-acetylglucosamine standards was carried out in parallel experiments. The estimated N-acetyl amino sugar contents were calculated for the dry weight of bacteria.

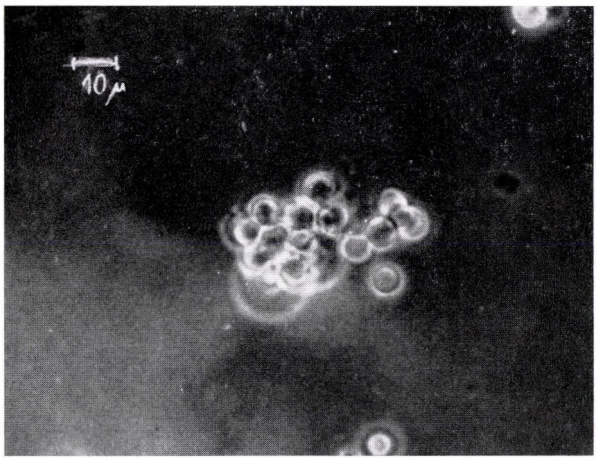

Fig. 1. Phase contrast micrograph of strain L_2 (15th subculture) grown in penicillin-free medium for 48 hours. Magnification, × 630

The N-acetyl amino sugar content of L strains derived from *Staph. aureus* 100 was determined for subcultures 4, 6, 8, 10 and 14 of the 3 freshly induced strains, and for a presumably stable L form (SL), which had been obtained from *Staph. aureus* 100 and passed through more than 100 subcultures in penicillin-free medium. The results are shown in Table I.

It is clear that strains L_2 and L_3 produced N-acetyl amino sugars in amounts about 5 times higher than SL and about 10 times higher than L_1.

The stability of subcultures of L forms was also checked. The cultures were seeded into penicillin-free medium 2; penicillin transferred with the inoculum was eliminated with penicillinase (Neutrapen, Riker Laboratories, Inc.). Incubation lasted for one week at 37°C. Reversion of the cultures was examined with a phase contrast microscope and culturing on blood agar plates. The results are presented in Table II.

From Table II it is seen that passing through 6 subcultures was sufficient to obtain irreversible forms from freshly induced strains L_1 and L_3. Strain L_2 which was more liable to reversion, also became fairly stable after the 14th subculture.

Table II

Reversion of Staph. aureus L forms in penicillin-free liquid medium

L strains	Reversion after 1 week's incubation at 37°C in subculture				
	4	6	8	10	14
L_1	+	—	.	.	.
L_2	+	+	—	+	—
L_3	+	—	—	—	—

. Not done.

In subsequent examinations it was attempted to determine the type of Park nucleotide yielding the found N-acetyl amino sugars. Mass cultivation of strains L_2, L_3, LS and *Staph. aureus* 100 was carried out in liquid medium 2. To the bacterial culture penicillin was added four hours before the end of cultivation (500 units/ml). The cultures were then treated as described above. After shaking with ether, part of the sample was used for N-acetyl amino sugar determination; the other part was concentrated *in vacuo* then nucleotides containing N-acetyl amino sugars were paper chromatographed as described by ITO and SAITO [12]. Nucleic acids were determined spectrophotometrically at pH 2, 7 and 10. Amino acids were detected by paper chromatography after hydrolysis in 6 *N* HCl for 24 hours.

In *Staph. aureus* 100 cultured for 20 hours in the absence, then for 4 hours in the presence, of penicillin, as well as in strains L_2 and L_3 cultured with penicillin from the beginning, an uridine diphosphate nucleotide (UDP-nucleotide) containing N-acetyl amino sugar, glutamic acid, glycine and lysine, occurred in higher amounts. UDP-nucleotides containing glycine and N-acetyl amino sugar and only acetyl amino sugar were also encountered.

In strain SL a small amount of acetyl amino sugar-containing UDP-nucleotide devoid of amino acids was revealed.

It was interesting that when the same *Staph. aureus* strain was cultured in the medium described by STROMINGER [9], and after 18 hours incubation it was exposed to penicillin (500 units/ml) for 30 minutes, it yielded 6 different UDP-nucleotides containing the following components: (1) N-acetyl amino sugar, lysine, glutamic acid, glycine, alanine; (2) N-acetyl amino sugar, lysine, alanine, glutamic acid; (3) N-acetyl amino sugar, glutamic acid, glycine; (4) N-acetyl amino sugar, aspartic acid; (5) N-acetyl amino sugar, aspartic acid, glutamic acid, glycine, lysine; (6) Only N-acetyl amino sugar. Amino acids have been determined qualitatively only, therefore the results do not refer to amino acid sequences. In addition, a nucleotide, containing small amounts of cytidine base, was demonstrated. It has been assumed that the observed high amount of N-acetyl amino sugar-containing uridine nucleotide is produced

Table III

N-acetyl amino sugar content of Staph. aureus L forms in penicillin-free and penicillin-containing liquid medium

Strain	N-acetyl glucosamine μg/mg dry weight	
	Without penicillin	With penicillin (500 μg/ml)
L_2 (15th suculture)	2.1	8.8
L_3 (15th subculture)	3.8	9.7
LS	2.0	1.8
Staph. aureus 100 bacterial form	2.4	9.8

only when strains L_2 and L_3 are cultured in the presence of penicillin. Therefore 15th penicillin-free medium 2 subcultures, which contained relatively stable L forms, were investigated. Penicillin transferred with the inoculum was neutralized with penicillinase. The results are shown in Table III.

It is evident that, as compared to strain LS, in the presence of penicillin strains L_2 and L_3 produced at least 5-fold amounts of N-acetyl amino sugar-containing nucleotides (Tables I and III). In penicillin-free medium strains L_2 and L_3 and *Staph. aureus* 100 accumulated the corresponding substance in considerably lower amounts.

Discussion

In order to elucidate whether L forms do or do not possess rigid cell walls characteristic of bacteria, detection of cell wall materials in stable and unstable L forms has been attempted by several authors. WEIBULL [14], MORRISON and WEIBULL [15], KANDLER *et al.* [16, 17] and MARTIN [18] revealed different amounts of cell wall materials in L forms of *Proteus mirabilis*.

In examining the amino sugar content of *Staph. aureus* H and *Streptococcus pyogenes* G—18, SHARP [6] found that, as compared to bacterial cultures, L forms contained very small amounts of amino sugar. In contrast, the L forms of *P. vulgaris* P—18 contained considerable amounts of bound amino sugars. SHARP therefore assumed that the cell wall synthesis in various L forms may be inhibited at different stages. A similar conclusion has been drawn by MARTIN [18].

The investigations of PARK and STROMINGER [1—5] have revealed the role of muramic acid-peptide-containing nucleotides in cell wall synthesis. As compared to the streptococcal parent, in the derived L forms EDWARDS and PANOS [19] found increased amounts of uridine diphosphate muramic acid peptides. This finding indicates that in streptococcal L forms incorporation into the cell wall of these substances is inhibited.

On the basis of these data we have examined the N-acetyl amino sugar content of staphylococcal L forms. As to the stage in which cell wall synthesis is blocked, our results indicate at least two variations (Tables I and III). In the presence of penicillin strains, SL and L_1 contained small, while strains L_2 and L_3 high amounts of N-acetyl amino sugar UDP-nucleotides.

When cultured in STROMINGER's medium [9], unlike *Staph. aureus* Copenhagen, our staphylococcal strain contained aspartic acid and glycine in the UDP-nucleotide precursors. The same amino acids were demonstrated in *Staph. aureus* 209P by ITO *et al.* [20, 21].

The present experiments have also shown that strains L_2 and L_3 accumulated mainly uridine-diphosphate muramic acid peptides consisting of UDP-N-acetyl amino sugar, glutamic acid, glycine and lysine. In strain SL this nucleotide was not demonstrated.

It has been concluded that in strains L_2 and L_3, corresponding to its known site of action, penicillin inhibits the incorporation of muramic acid peptide. In strains SL and L_1 the amount of N-acetyl amino sugar-nucleotide does not increase in the presence of penicillin. In strains L_2 and L_3 cultured in penicillin-free medium, the amount of uridine nucleotide precursors decreases. That this finding cannot be attributed to a newly started cell wall synthesis is indicated by microscopic and cultural control examinations (Table I, Fig. 1).

The findings that freshly induced (L_1) and stable (SL) L forms show small degree of Park nucleotide synthesis in penicillin-containing medium, and that other freshly induced L forms (L_2, L_3) produce 5 times higher amounts of Park nucleotides in the presence than in the absence of penicillin, seem to confirm the conception of MATTMANN *et al.* [22]. According to this theory in the development of staphylococcal L forms penicillin plays merely a selective part and L forms are produced from mutants incapable of cell wall synthesis. The site of the block is not critical. Therefore the term L form refers to a population in which different stages of cell wall synthesis are inhibited. This conception has been supported by FJEDOROVA [23], who demonstrated that enzymic digestion of the cell wall highly increases the L form production in *Str. pyogenes* and *S. typhi*. The appearance of bacterial forms when the first L subcultures are seeded into penicillin-free medium does not disprove this hypothesis, because the reversion may be due to the multiplication of some mutants capable of cell wall synthesis.

The other possibility is that the antibiotic acts specifically. Cell wall synthesis is blocked at the site characteristic of penicillin action. In subsequent derivatives a genetic fixation of the blockade may occur; thus cell wall synthesis will be inhibited at the same stage, even in the absence of penicillin. This, however, seems improbable, since in penicillin-free 48 hour cultures the accumulation of uridine nucleotide precursors considerably decreases (strains L_2 and L_3). There is no normal cell wall synthesis; plating on blood

agar and microscopic examination fail to reveal any sign of reversion into bacterial forms.

It is more likely that in some staphylococcal L forms the genetic block of cell wall synthesis is located beyond the site of penicillin action. Elucidation of the problem will be attempted in further experiments.

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STUDIES OF THE AEROBIC ENTERIC FLORA OF INFANTS

By

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(Received February 25, 1965)

Summary. Examination of 6890 faecal samples has shown that, except for Staphylococci, aerobically cultivable facultatively pathogenic bacteria occurred practically at the same frequency in infants with and without enteric symptoms. No significant difference was revealed as to the incidence of "dysbiotic" faecal samples in which the number of these organisms exceeded the usual relative coli counts. Facultative pathogens identified more precisely by serological and biochemical methods were also evenly distributed between the two groups of infants.

It is known that only a small part of enteric infections occurring among infants under one year of age can be attributed to classical pathogenic bacteria such as Shigellae, Salmonellae and certain *E. coli* serotypes. Elucidation of the aetiology of cases with negative bacteriological findings has been attempted by several authors. Some of them have concluded that the present methods are not adequate for detecting the pathogen in a considerable part of the materials. Other investigators have assumed the aetiological role of viruses, metabolic disorders and, last but no least, of facultatively pathogenic enteric bacteria [1—3], the significance of which bacteria has recently been stressed by clinicians [4]. Owing to the frequent nosocomial occurrence of facultative pathogens, examination of hospital material cannot yield exact data as to the distribution of these organisms among the population. It seemed therefore advisable to make a survey of our large routine material originating from non-hospitalized infants.

Materials and methods

Faecal samples were streaked on plates of bismuth sulphite, desoxycholate citrate, brilliant green, Endo, blood agar and nutrient agar media. Selenite and rhodanate [5—6] enrichment media and Sabouraud medium were also used. Thus, in addition to Salmonellae, Shigellae and pathogenic coli bacteria, all aerobically cultivable microorganisms occurring in faeces could be detected.

With the exception of *E. coli* producing morphologically typical lactose positive colonies on Endo agar, all isolates were classified by biochemical reactions. Salmonella, Shigella, pathogenic *E. coli* and *Proteus hauseri* strains were serotyped. Biochemical differentiation was based on the indole, urea, hydrogen sulphide and KCN reactions. In addition to all fermentation tests recommended by SEDLÁK [7], the methyl red, Voges-Proskauer, phenylalanine deaminase, citrate and malonate reactions were also performed.

Staphylococcal strains were phage-typed in our Phage Laboratory; yeast-like organisms were classified in our Mycological Laboratory. The specimens were grouped according to the physician's record on the report form as originating from infants suffering from enteric disease and from infants free of enteric symptoms.

Results

Our examinations carried out in the years 1963—1964 included 6890 faecal specimens. Of the samples 4320 were obtained from non-hospitalized infants with enteric symptoms; 1133 samples were obtained in course of the compulsory examination of healthy infants entering nurseries. The age

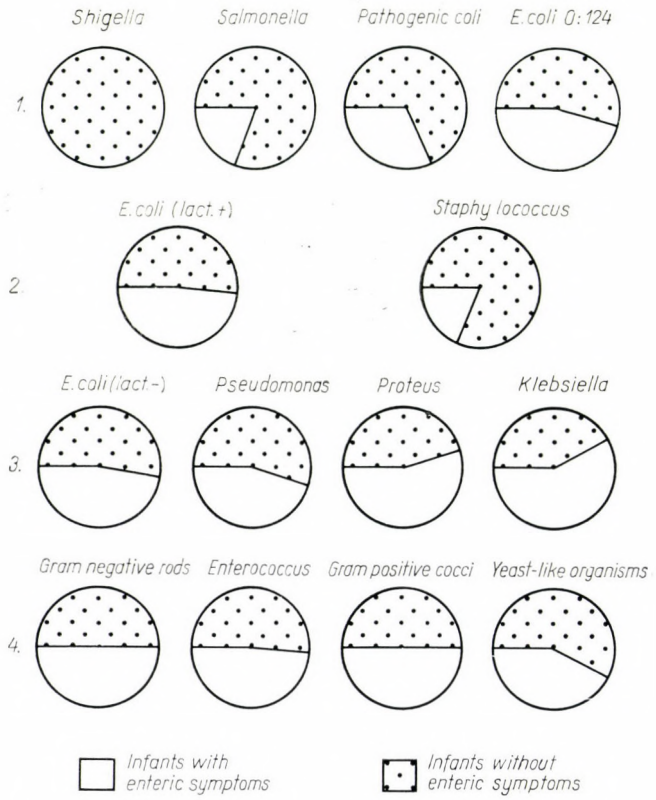

Fig. 1. Comparison of the incidence of aerobically cultivable microorganisms in infants with and without enteric symptoms

of each member of both groups was under one year. In addition faecal specimens from 1437 healthy adults (mainly food handlers) were also examined.

Table I shows the incidence of various microorganisms in the three groups. The data include all organisms detectable without enrichment on aerobically incubated plated media, regardless their absolute counts in the specimens. It is seen that, especially as to the incidence of *E. coli*, the enteric flora of infants and adults differed considerably. *E. coli* strains fermenting lactose within 24 hours were more prevalent in adults than in infants. In contrast, late-lactose-fermenting or lactose negative *E. coli* was more frequent

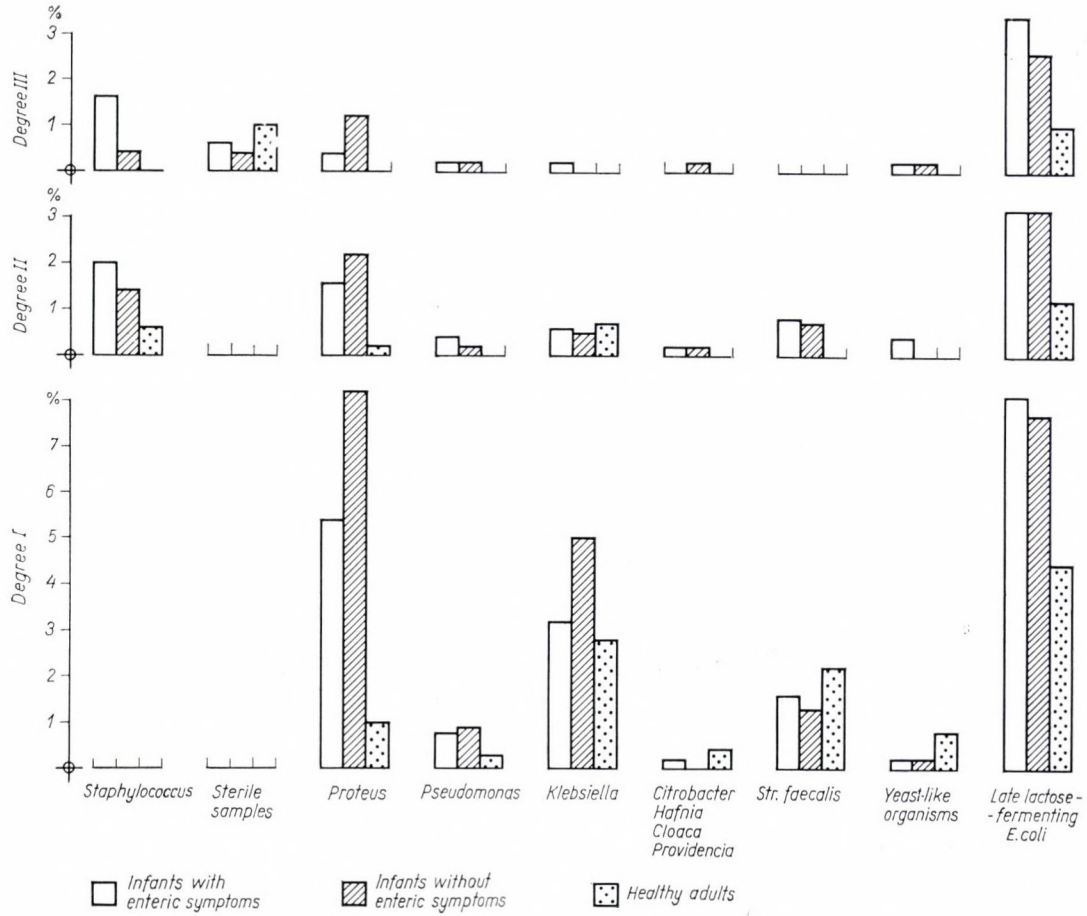

Fig. 2. Incidence of different degrees of dysbiosis in faecal samples

Table I

Percentage distribution of aerobically cultivable organisms in faecal samples

	Infants with enteric symptoms	Infants without enteric symptoms	Healthy adults
No. of samples	4320	1133	1437
Salmonella	2.6	1.5	0.7
Shigella	0.8	—	0.2
E. coli O 124	0.7	0.6	0.9
E. coli associated with infantile enteritis	4.8	2.5	1.3
Staphylococcus aureus	3.9	2.2	0.6
Proteus	9.3	11.9	1.9
Pseudomonas aeruginosa	1.8	1.5	0.2
Klebsiella	4.7	6.5	2.1
Cloaca	0.02	—	0.06
Hafnia	0.06	—	—
Citrobacter	—	0.2	—
Providencia	0.02	—	—
Streptococcus faecalis	3.7	4.0	3.9
Streptococcus viridans	0.09	0.1	0.4
Streptococcus pyogenes	0.2	—	0.5
Staphylococcus albus	0.5	0.9	1.0
Gram positive cocci	0.4	0.8	1.0
Yeast-like organisms (blood agar)	0.5	0.2	0.2
Spore-bearers	0.7	0.4	6.3
E. coli (lactose negative)	17.8	16.5	8.6
E. coli (lactose positive)	55.7	53.8	77.3

in infants. While members of the Proteus, Staphylococcus, Pseudomonas and Klebsiella groups were fairly common in infants, healthy adults harboured these organisms only occasionally. *Str. faecalis* was encountered in an even distribution. Less common enteric bacteria (Citrobacter, Hafnia, Cloaca, Providencia) were infrequently met with.

Fig. 1 compares the incidence of various microorganisms in infants with and without enteric disease. Diagrams in the upper row show that bacteria unanimously recognized as pathogenic, occurred mainly in the group of infants with enteric symptoms. Facultatively pathogenic bacteria indicated in row 3 occurred at an approximately equal frequency in both groups. The incidence of quick-lactose-fermenting *E. coli* was similar to the former organisms; the distribution of *Staph. aureus* resembled that of the obligatory pathogens (row 2).

Fig 2. shows the incidence of faecal samples in which bacteriological findings were indicative of dysbiotic conditions. The data were based on the relative number of colonies of facultative pathogens and of quick-lactose-fermenting *E. coli* on Endo and blood agar plates. When examinations carried out in this manner suggest dysbiosis, many Hungarian public health laboratories classify the result into 3 degrees: degree I indicates the presence of moderately, while degree II that of considerably increased numbers of organisms other than quick-lactose-fermenting *E. coli;* in faecal samples falling into degree III these organisms comprise the overwhelming majority [8].

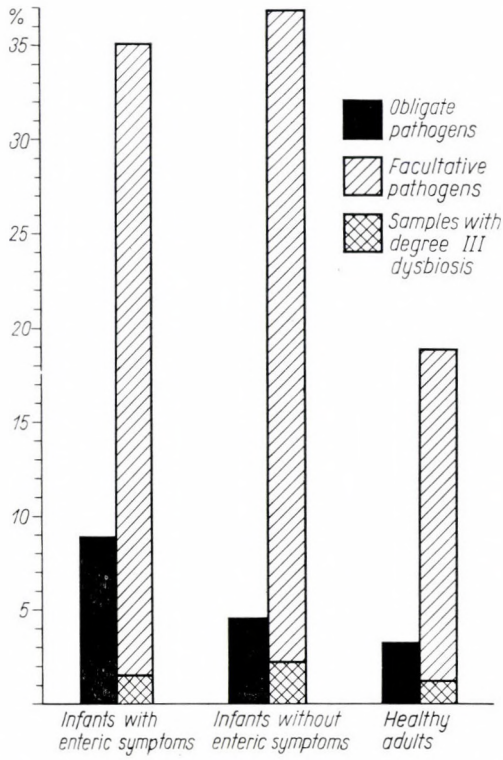

Fig. 3. Incidence of obligatory and facultative pathogens in faecal samples

Fig. 3 summarizes the percentage distribution of obligatory and facultative pathogens in the three groups. Samples with degree III dysbiosis are indicated separately in the columns representing the incidence of facultative pathogens. Such samples were infrequent among infants with enteric infections, symptomless infants and healthy adults (1.5, 2.3 and 1.2 per cent, respectively).

The percentage distribution of bacteria isolated from 1 to 8-week old infants with enteric symptoms is separately shown in Fig. 4. From the faeces of this group no obligatory pathogenic enteric bacteria were revealed. The

Table II

Results obtained with blood agar and rhodanate enrichment medium

Examination group	No. of samples	Per cent positive on blood agar		Per cent positive in rhodanate medium	
		Staphylo-coccus	*Str. faecalis*	Staphylo-coccus	*Str. faecalis*
Infants with enteric symptoms	673	5.0	6.2	57.8	57.0
Infants without enteric symptoms	372	2.6	6.0	52.6	59.1
Healthy adults	222	0.9	5.9	6.3	33.7
Total	1267	3.6	6.1	55.1	53.5

incidence of Staphylococci in this group was almost 10 times higher than in older infants of the same examination group. *Ps. aeruginosa*, Proteus and Klebsiella also occurred at considerably higher frequencies than the average for the total material.

Evaluation of the rhodanate enrichment medium [5, 6] for the isolation of Staphylococci and *Str. faecalis* has shown that by use of this medium the number of positive findings was 10 times higher than by culturing on blood agar (Table II). Of the isolated *Staph. aureus* strains 28 per cent were typeable with "additional" phages; 23 per cent of these cultures fell into phage type 42 D/1, first isolated by KENDE during a hospital outbreak [9]. By the use of Sabouraud's medium yeast-like organisms were revealed in all groups approximately in 30 per cent. About 50 per cent of the isolates belonged to *Candida albicans;* the remaining strains represented 43 different species and

Table III

Distribution of Proteus hauseri serogroups

Serogroup	Number of isolated strains			
	Infants with enteric symptoms	Infants without enteric symptoms	Healthy adults	Total
0 3	81	34	7	122
0 6	20	11	—	31
0 10	20	7	2	29
0 13	36	5	1	42
0 26	8	1	2	11
0 28	6	3	—	9
Other	212	69	15	296
Total	383	130	27	540

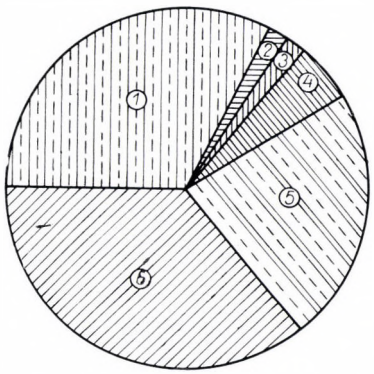

Fig. 4. Percentage distribution of bacteria isolated from infants under 8 weeks of age. 1, Staphylococcus; 2, Proteus; 3, Klebsiella; 4, Pseudomonas; 5, *E. coli* lactose negative; 6, *E. coli* lactose-positive

were evenly scattered among all examination groups. Serological typing of *P. hauseri* was performed with 0 sera 3, 6, 10, 13, 26 and 28. The results were in agreement with those of LÁNYI [10], as our material yielded most frequently serogroup 3 cultures. However, there was no significant difference as to the incidence of Proteus serogroups in infants with and without enteric disease (Table III).

Discussion

The purpose of the present survey has been to estimate in a large routine material the aetiological role in infantile enteritis of facultatively pathogenic organisms. In 6890 faecal specimens obligatory pathogens were isolated more frequently from infants with enteric infection than from the control groups. The pathogenicity of Shigellae was obviously demonstrated: all Shigella strains occurred in the former group. Salmonellae and *E. coli* serotypes associated with infantile enteritis were more prevalent among infants with enteric symptoms. On the other hand, there was no significant difference in the distribution of facultative pathogens among the examination groups. Only the incidence of Staphylococci was similar to that of the obligatory pathogens (Fig. 1).

The number of facultative pathogens in the faecal sample as related to the coli count is frequently considered as an indicator of normal or dysbiotic conditions. Therefore, when facultative pathogens overgrow quick-lactose-fermenting coli colonies, the finding and perhaps the antibiogram of the incriminated organism is reported; whether the organism can or cannot be associated with the clinical symptoms is, however, left to the clinician's consideration. In the present investigations, dysbiotic faecal samples were grouped according to the 3 degree classification method [8]. Fig. 2 demonstrates that, except when Staphylococci appeared in unusual numbers, there was no signif-

icant difference as to the occurrence of dysbiosis in various groups of the examined material. Even "sterile" specimens, from which aerobically cultivable bacteria were absent, occurred at about the same frequency in infants with and without enteric symptoms. It was striking that dysbiotic samples were fairly common in all examination groups (Fig. 3). This finding confirms the concept that facultative pathogens are normal members of the enteric flora [11]. Figs 2 and 3 demonstrate that the presumably more significant degree III dysbiosis was quite rare (1 to 2 per cent). The statistical lack of significance of facultative pathogens in the aetiology of enteric infections is obvious if the incidence of obligatory pathogens is compared to the incidence of degree III dysbiosis (Fig. 3).

It is generally accepted that dysbiotic conditions may partly be attributed to antibiotic treatment. In the present investigations dysbiosis was common in the control group of infants examined before admission to the nursery; it is improbable that these infants had received antibiotics. Thus our findings suggest that the presence of facultative pathogens cannot always be associated with antibiotic treatment.

The enteric flora of infants with enteritis under 8 weeks of age differed from that of older infants (Fig. 4). This finding may be due to the fact that the former infants still harboured Staphylococcus, Proteus, and *Ps. aeruginosa* which they had acquired in the newborn babies' wards.

The present investigations indicate that, at least at present, the classification of enteric bacteria into obligatory pathogenic and non-pathogenic (facultatively pathogenic) organisms is justified. It cannot be denied, of course, that under certain conditions (injured mucosa or changes in the eubiotic flora) some facultatively pathogenic organisms may occasionally induce or maintain more severe enteric symptoms than those produced by obligatory pathogens.

Acknowledgement. The author is indebted to *Mrs.* I. VÁLY for her help in collecting statistical data.

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VIROLOGICAL INVESTIGATION OF HOSPITALIZED CASES OF PSEUDOCROUP AND ACUTE LARYNGOTRACHEOBRONCHITIS

By

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(Received March 15, 1965)

Summary. Infants and young children with the pseudocroup — acute laryngotracheobronchitis syndrome, admitted during a period of 12 months were tested for respiratory viruses. Virus isolation was attempted in 423 cases, antibody response was determined in 229 serum pairs.

Twohundred and sixty-one virus strains were isolated in the following percentage distribution: parainfluenza type 3, 34.1 per cent; RS virus 23.0 per cent; adenovirus 17.6 per cent; parainfluenza type 2, 16.1 per cent; unidentified haemadsorbing agent 5.7 per cent; enterovirus 1.9 per cent; parainfluenza type 1, 1.1 per cent.

Aetiological diagnosis could be based on both virus isolation and serological test in 128 cases, on virus isolation alone in 133 cases, on serological test alone in 58 cases.

The immunological relation among parainfluenza types, the double immune responses, the problem of recurrent pseudocroup, and the aetiology of complicated cases are discussed.

The use of tissue cultures in virus research and the introduction of the haemadsorption technique [1] have led to the discovery of numerous respiratory viruses [2—7].

Among the newly-recognized viruses, types 1—3 of the parainfluenza viruses and the respiratory syncytial (RS) virus have proved to be the most common pathogens in the acute respiratory diseases of infants and young children.

The term pseudocroup (Ps)-acute laryngotracheo-bronchitis (ALTB) syndrome characterizes an acute and thoroughly investigated respiratory condition [8—11]. Recent investigations have shown that the Ps—ALTB syndrome is most frequently associated with parainfluenza virus infection, but some other viruses (RS virus, adenoviruses) may also have an aetiological role. The present work was undertaken to contribute Hungarian data to the knowledge concerning the aetiology of the Ps—ALTB syndrome.

From the end of November, 1963, to the end of November, 1964, samples taken from 423 infants and young children treated at the Otolaryngological Section of this Hospital were tested for respiratory viruses. From the same group of children 229 paired sera were tested serologically.

Materials and methods

Specimens. Pharyngeal secretion was taken at admission by means of a sterile aspirator into Parker's 199 containing 1 per cent egg albumin and antibiotics. The specimens were centrifuged at 8000 r. p. m. and 2000 units of penicillin, 2 mg of streptomycin and 100 units of

mycostatin per ml, were added. With regard to the sensitivity of RS virus to freezing [12, 13], the samples were stored at +4° C and the inoculations were made as soon as possible. From several patients laryngeal secretion was also taken. These specimens were prepared in the same way as the pharyngeal secretion.

The first blood sample was taken on the day of admission, the convalescent-phase sample 14—16 days later, in several cases at the end of the third week. The sera were heated at 56°C for 30 minutes and kept at —20°C until used.

Tissue cultures. Monkey-kidney (MK) primary cell cultures were prepared as recommended by YOUNGNER [14]. The continuous cell line MK No. III/1 was obtained from Dr. RUZICSKA [15]. As maintenance fluid Parker's 199 was used for both primary and continuous MK cells. The maintenance fluids for HEp-2 and HeLa cultures consisted of 10 per cent calf serum in Parker's 199, and 10 per cent rabbit serum and 0.5 per cent lactalbumin hydrolysate, respectively, in Hanks' solution.

The cell lines were those maintained in the State Institute of Hygiene, Budapest.

Immune sera. Anti-parainfluenza and anti-RS sera were prepared in rabbits as recommended by DÖMÖK *et al.* [16]. The strains parainfluenza 1 Cop. 222; parainfluenza 2 ALTB paraflu; parainfluenza 3 Moss V 1020 and RS Long, each cultivated in primary MK cells, were used for immunization. The enterovirus and adenovirus typing sera were kindly supplied by the State Institute of Hygiene, Budapest.

Virus-isolation experiments. The onehundred and fifty-eight specimens obtained in the early phase of the present studies, were inoculated in parallel into primary MK and HEp-2 cell cultures, 0.1 ml per tube. From both kinds of culture three tubes were inoculated with each specimen. With specimens obtained later, only primary MK cells were inoculated. After inoculation the tubes were incubated at 37°C in a stationary position for 20 days. The cultures were controlled microscopically on every second day. Each material was submitted to 2 or 3 passages. Irrespective of the appearance or absence of cytopathic effects (CPE), haemadsorption (HAds) tests were performed at intervals. For the latter purpose one culture of those inoculated with the same material was washed with Parker's 199, and 0.2 ml of a 1 per cent suspension of guinea-pig erythrocytes was added to each culture. The tubes were incubated in the horizontal position at room temperature for 30 minutes. The cultures were subsequently washed three times with Parker's 199, and examined under the microscope. With regard to a possible infection of the cultures by SV-5, SV-41 [17, 18] or any other "spontaneous" virus, a number of control cultures were tested simultaneously.

Identification of isolates. The cultures showing the HAds and CPE characteristic of parainfluenza viruses [2—4] and those showing only HAds were frozen and thawed three times and then tested for haemagglutination (HA) with a 1 per cent suspension of human and guinea-pig erythrocytes. The test was carried out in TAKÁTSY's Microtitrator apparatus [19] at room temperature. The strains thus classifiable as Myxoviruses were typed in primary MK cell cultures by the HAds-inhibition test using anti-parainfluenza rabbit sera.

The isolates forming syncytia in HEp-2 and/or primary MK cell cultures without showing HAds were identified by the neutralization test carried out with anti-RS serum in primary MK cell culture.

In the neutralization test the inoculum contained 100 CPD_{50} in a volume of 0.1 ml. The virus-serum mixtures were incubated at 37° C for 1 hour. Final reading of the test took place after an incubation for 5—7 days (parainfluenza viruses) or 7—10 days (RS virus).

The medium obtained from primary MK, or HEp-2 cultures suspect of adenovirus on the basis of the CPE was submitted to a passage in HeLa cell cultures. The cultures showing typical degeneration were frozen and thawed three times and then tested with the group-specific adenovirus complement-fixing (CF) antigen. Only five of the adenovirus isolates were typed by the neutralization test.

When CPE characteristic of enteroviruses was observed, the isolates were identified by the neutralization test in primary MK cell cultures.

Serological tests. CF tests were carried out with S antigens prepared from influenza A (PR8), influenza A-2 (Singapore 1/57), influenza B (Bon), adenovirus (a mixed antigen prepared from types 1—4, 7 and 15) and mumps virus (Enders) and with RS virus antigen. The antigens except the RS antigen were kindly supplied by the Virus Section of the State Institute of Hygiene, Budapest. The RS antigen was prepared by us in HEp-2 and primary MK cultures according to the prescription of ROSS *et al.* [20].

HA inhibition (HI) test was carried out with the Sendai strain of type 1 parainfluenza virus. The sera were absorbed with kaolin and guinea-pig erythrocytes [21] and then tested against 4 HA units of virus.

Both CF and HI tests were carried out in TAKÁTSY's Microtitrator in two-fold serum dilution series.

For neutralization tests with human sera, serum-virus mixtures were prepared to contain the given serum dilution and 100 CPD_{50} per 0.1 ml. The mixtures were incubated at 37°C for 1 hour. Three tube cultures were inoculated with each mixture. The neutralization tests for parainfluenza 2 and 3 antibodies were carried out in continuous MK cell cultures, those for the RS virus in primary MK and HEp-2 cultures.

Results

From the 423 pharyngeal and laryngeal secretions 261 virus strains were isolated. The distribution of the strains was as follows.

Table I

Distribution of isolates by type and the age of patients

		Age								Total
		Months		Years						
		0–5	6–11	1	2	3	4	5–6	7–10	
No. of children tested....		4	57	102	117	67	30	16	30	423
Positive	No.	2	37	65	71	45	19	10	12	261
	%	50	65	64	61	67	63	62.5	40	61.7
Isolates										
Parainfluenza 1		—	—	1	2	—	—	—	—	3
Parainfluenza 2		1	4	9	8	11	4	4	1	42
Parainfluenza 3		—	9	26	28	13	9	2	2	89
Unknown HAds.		1	4	3	3	2	—	1	1	15
RS virus		—	9	15	14	11	4	2	5	60
Adenovirus		—	9	9	16	6	2	1	3	46
Enterovirus		—	2	1	—	2	—	—	—	5
Unidentified		—	—	1	—	—	—	—	—	1

Parainfluenza 3 virus, 89 (34.1 per cent); RS virus, 60 (23.0 per cent); adenovirus, 46 (17.6 per cent); parainfluenza 2 virus, 42 (16.1%); unidentified haemadsorbing strains, 15 (5.7 per cent); enterovirus, 5 (1.9 per cent); parainfluenza 1 virus 3 (1.1 per cent); unidentified agent, 1 (0.4 per cent).

From the pharynx of one patient parainfluenza type 3 and an adenovirus were isolated simultaneously.

Out of the 46 adenovirus strains only five were typed. All proved to be of type 3. The five enterovirus strains were identified as echovirus 8, echovirus 11, coxsackievirus B-2, coxsackievirus B-3 and poliovirus type 2, respectively.

Out of the 423 patients 103 had relapses. From these 59 strains were isolated, namely 24 of parainfluenza type 3, 14 of adenovirus, 13 of RS virus, 6 of parainfluenza type 2, one unidentified haemadsorbing agent, and a cox-

sackievirus B-3 strain. This distribution agrees well with the distribution of the strains isolated from the primary cases with the Ps—ALTB syndrome.

The distribution of the cases and the isolates by age is shown in Table I.

The isolation rate showed no significant age-dependence. Sixty-five per cent of the patients was 6 to 35 months of age, and 66 per cent of the isolates originated from the same age-group. The age distribution of the different

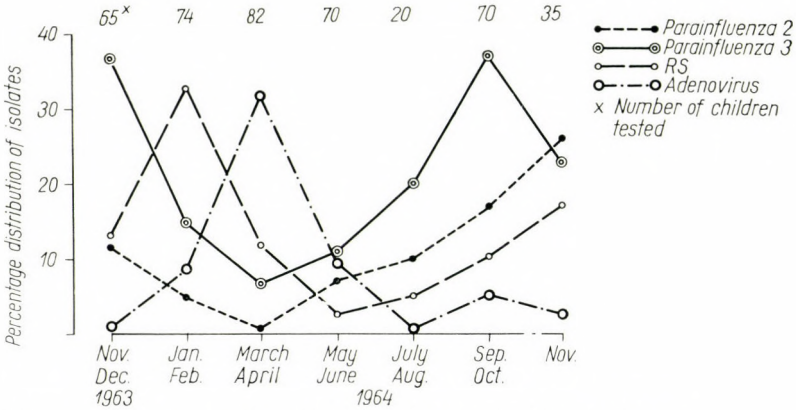

Fig. 1. Seasonal variation of the incidence of different viruses

viruses was consistent with the general distribution. The low incidence of parainfluenza viruses as compared to the RS and adenoviruses over 7 years of age could not be appreciated in view of the small number of isolations in this age group.

During the period of investigation the incidence of the different viruses (or types) showed a considerable seasonal fluctuation (Fig. 1).

The curve representing the frequency of parainfluenza 3 (as expressed in per cent of the total number of isolates during the same period) started at 37 per cent, then tended downwards until March—April (7 per cent). In May—June it began to rise again to reach a peak near the starting level in September—October. The shape of the frequency curve of the parainfluenza type 2 infections was similar, but the incidence of this virus was lower than that of parainfluenza type 3 in every season, except in November, 1964.

The sudden appearance of the RS virus in the form of intensive epidemics is well-known [22, 23]. Such an epidemic wave was observed during our studies in January and February, when RS virus was isolated from 33 per cent (27 cases) of the cases yielding virus. The curve fell to a bottom level in May—June and showed a slow rise during the autumn months.

Surprisingly, the course of the adenovirus curve was very similar to that of the RS curve, except that its peak was shifted to March and April. The echovirus type 8 and 11 strains were isolated in February and April, respec-

Table II

Isolation of viruses from children, classified by clinical symptoms

Clinical picture	Cases tested		Cases yielding virus		Numerical and percentage distribution of isolates															
					Parainfluenza						RS		Adeno		Unknown haemadsorpt.		Entero		Unidentified	
					1		2		3											
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Pseudocroup	57	135	33	58	1	3	5	15	9	27	7	21	10	30	1	3	—		—	
Pseudocroup mild laryngitis	234	55.3	140	60	1	1	22	16	54	39	30	21	23	16	8	7	2	1	—	
Pseudocroup moderate laryngitis	62	14.7	45	73	1	2	7	16	15	33	12	27	6	13	3	7	1	2	—	
ALTB	6	1.4	4	67	—		2	50	1	25	—		1	25	—		—		—	
Undetermined	64	15.1	39	61	—		6	15	10	26	11	28	6	15	3	8	2	5	1	3
Total	423	100.0	261	62	3	1.1	42	16.1	89	34.1	60	23	46	17.6	15	5.7	5	1.9	1	0.4

tively, whereas the two coxsackievirus B strains during the enterovirus season, in July.

We attempted to categorize our isolates according to the clinical manifestations. Based on clinical and anamnestic data, 359 of the 423 cases under study were classified into four clinical groups.

Group 1. Classical Ps. Afebrile cases without inflammatory symptoms. In these cases the Ps syndrome seemed to be a manifestation of a general allergic condition. This group included 57 cases.

Group 2. Ps syndrome with mild laryngitis and fever (234 cases).

Group 3. Ps syndrome with moderate laryngitis and fever (62 cases).

Group 4. Severe ALTB (6 cases; 4 of these were tracheotomized).

In 64 cases the anamnestic data were insufficient for clinical classification.

Table II shows the distribution of the isolates by clinical groups. It is seen that the general isolation rate ranged from 58 per cent (group 1) to 73 per cent (group 3) in the different categories. This difference was too little to suggest a correlation between the presence of detectable virus and the inflammatory manifestations.

Serological studies

The serological tests were performed in order to extend the aetiological evidence based on virus isolation and to contribute further data to the antigenic relationships among parainfluenza viruses as well as to the debated question of the specificity of positive serological findings in connection with respiratory virus infections.

The numerical data of the serological examinations carried out with the different antigens and the numerical and percentage data of the paired sera that proved to be positive in the different tests are presented in Table III.

Table III

Serological tests in 229 serum pairs

		CF						HI	Neutralization**		
		Influenza			Adeno	Mumps	RS	Sendai	Parainfluenza		RS
		A_0	A_2	B					2	3	
Number of serum pairs tested		229	229	229	229	71	91	167	214	215	31
Positive*	No.	—	—	4	33	6	—	4	41	110	22
	%	—	—	1.7	14.4	8.4	—	2.4	19.2	51.2	71

* = at least fourfold rise in titre during illness.

** = Each of three children who had yielded echovirus 8 and 11 and coxsackievirus B—3, respectively, gave antibody response to the homologous type.

Out of the 229 serum pairs available 149 had been obtained from children from whom virus was isolated. In 128 serum pairs we were able to demonstrate a homologous antibody response. An at least fourfold rise in the homologous neutralization titre was demonstrated in 72, 24, 22 and 3 cases from which parainfluenza type 3, parainfluenza type 2, RS, and enterovirus, respectively, were isolated. Out of the 28 patients yielding adenovirus only 7 developed CF antibodies to the adenovirus group antigen.

In the remaining 80 cases we have attempted to establish the diagnosis by serological methods only. In 38 and 26 cases a rise in the neutralization titre to the parainfluenza type 3 and 2 viruses, respectively, was demonstrated. In 26 cases the adenovirus CF titre showed an at least fourfold rise, whereas in 22 cases no appreciable rise to any of the viruses under study could be demonstrated.

For the cases whose paired sera were tested for neutralizing antibodies to parainfluenza types 2 and 3 the data for antibody response and virus isolation are presented in Table IV.

Table IV

Comparison of virus isolation and homologous antibody response

(a) parainfluenza 2

Neutralization test		Virus isolation				Total	
		Positive		Negative			
		No.	%	No.	%	No.	%
Positive	No.	24		16		40	
	%		11.2		7.5		18.7
Negative	No.	1		173		174	
	%		0.3		81.0		81.3
Total		25	11.5	189	88.5	214	100

(b) parainfluenza 3

Neutralization test		Virus isolation				Total	
		Positive		Negative			
		No.	%	No.	%	No.	%
Positive	No.	72		38		110	
	%		33.5		17.5		51
Negative	No.	—		105		105	
	%		—		49.0		49
Total		72	33.5	143	66.5	215	100

According to these data, examination of paired sera with the neutralization test appears to be more sensitive than virus isolation for demonstration of each of parainfluenza 2 and 3 infections. However, the high incidence of double serological reponses have rendered the diagnostic value of the former method questionable.

From relapsing patients with the Ps or ALTB syndrome 43 serum pairs were tested, mostly from virus-negative cases. A rise in titre to either of the viruses under study was demonstrated in 34 cases.

Comparison of the antibody titres obtained by different tests. In the first samples of primary cases no neutralizing antibodies to parainfluenza type 2 virus were found, except in low titres (1 : 8 or 1 : 16) in the sera of several infants. These titres may be considered to be maternal in origin. In the convalescent samples, on the other-hand, 1 : 128, 1 : 256 and higher titres were common. Thus, the antibody responses were highly significant. In some of the recurrent cases low antibody titres were found in the acute-phase sera.

Antibodies, presumably of maternal origin, neutralizing parainfluenza 3 virus were often demonstrated in the acute-phase serum samples of infants. These low titres soon fell below 1 : 4 as shown by the relapsing patients whose antibody level was followed over a longer period of time. Over two years of age, however, hardly any serum was found to be negative. In the cases yielding parainfluenza 3 virus the convalescent titres attained or exceeded the 1 : 256 level. In some relapsing patients this virus was isolated in spite of the presence of homologous neutralizing antibodies in titres from 1 : 32 to 1 : 64.

The anti-RS neutralizing antibody was titrated first of all in the cases from which the virus was isolated. Among the acute-phase samples only two contained antibodies. In the convalescent sera the titres did not usually surpass the 1 : 64 value, *i.e.* did not reach the values seen in connection with parainfluenza types 2 and 3.

The highest homologous titres (1 : 512 to 1 : 1024) were reached in the cases which had yielded enteroviruses.

CF tests. A positive result to influenza A or A_2 antigens was never obtained. It should be noted that during the period of investigation, except for several institutional outbreaks caused by the A_2 virus in April 1964, influenza was not observed in Hungary. With the influenza B antigen, a rise in the CF titre was demonstrated in four cases.

Antibody response to the adenovirus group antigen was more frequently demonstrated in cases of recurrent Ps—ALTB than in primary cases.

With the RS antigen only very low, if any, CF titres (1 : 8 to 1 : 16) were demonstrable. None of the 91 paired sera tested showed any CF antibody response to this virus.

Antigenic relation within the parainfluenza group. The CF test with mumps antigen was carried out in 71 serum pairs obtained from patients who had

yielded parainfluenza virus. Antibody response was demonstrated in 6 cases; in each of them the parainfluenza type 3 titres were also increased. All the patients showing a double response were older than five years of age.

To investigate the relation among the parainfluenza types, 167 serum pairs were tested for HI antibodies to the Sendai virus. Nearly 60 per cent of the sera contained antibody up to 1 : 16 titre, but an appreciable rise in titre was demonstrated in as few as 4 cases. In further 33 cases a twofold rise was found. In these cases parainfluenza or RS infections were diagnosed.

Considering an at least fourfold increase in titre as positive, a double antibody response was demonstrated in 38 serum pairs. The commonest combinations were as follows.

adeno (CF)	+ parainfluenza 3 (neutr)	16 cases
adeno	+ parainfluenza 2	8 cases
adeno	+ RS (neutr)	2 cases
mumps	+ parainfluenza 3	6 cases

It should be noted that the HI test to the Sendai virus showed a twofold increase in 30 cases when parainfluenza 3 infection was demonstrated.

Complications

In some cases the classical clinical pattern of Ps or ALTB was followed by complications. In 10 cases spastic bronchitis, in 7 cases bronchopneumonia, in 13 cases severe laryngeal oedema, in 8 cases otitis media, in two cases tonsillitis and in one case maxillary sinusitis was observed.

Besides the virological examination, every patient was examined bacteriologically by the Bacteriological Laboratory of this hospital. In 44 cases the bacteriological findings were positive.

A comparison of the results has shown that for most of the complications the isolated virus was responsible. From cases of spastic bronchitis parainfluenza type 3 virus, from bronchopneumonia RS virus, adenovirus and parainfluenza type 2 virus were isolated. It was surprising that from each of the 8 cases complicated by otitis, parainfluenza type 2 virus was isolated.

In two cases Ps was observed during the incubation period of measles.

Discussion

Among the viruses isolated in the course of the present study from patients with the Ps—ALTB syndrome 51.3 per cent were identified as parainfluenza virus. This percentage agrees well with that published by McLean *et al.* [9], who isolated viruses in Toronto in the years 1960—1961. The predominance of parainfluenza viruses with the Ps syndrome has been shown in

some other countries as well (CHANOCK *et al.* [24], LEWIS *et al.* [8], BUKRINSKAYA and BLUMENTAL [10], FERRIS [25]).

In these wide-scale studies the incidence of parainfluenza type 1 was the highest. In the present studies, however, the incidence of this type of virus was strikingly low (1.1 per cent), whereas parainfluenza type 3 virus was isolated in an unusually high percentage (34 per cent).

Obviously, geographical and seasonal factors and the actual epidemiological situation may significantly influence the predominance of one or another type of virus [25]. It is also possible that the low incidence of parainfluenza type 1 virus was characteristic of the period of investigation in Budapest.

In the present material besides the unusual high incidence of parainfluenza type 3 and the relatively high participation of RS virus and of adenoviruses should be commented upon. It has been reported by several authors that the Ps syndrome may be caused by parainfluenza type 2 [2, 3, 8—10, 24, 28], parainfluenza type 3 [8—10, 25—27], certain adenovirus types [26, 28—30], the RS virus [26, 31] and a few types of echovirus [32], but the relative incidence of these viruses was usually low.

The unusual findings might have been due to the special composition of our clinical material, namely by the mildness of most of the cases. In contrast, our knowledge about the aetiology of the Ps—ALTB syndrome is based mainly on the examination of severe cases of ALTB.

On the other hand, the type distribution of the viruses isolated during the present work was very similar to that of the viruses recovered by PARROTT *et al.* [26], from cases with the pharyngitis-bronchitis syndrome. It might be supposed that the viruses causing mild Ps are, as a rule, different in type distribution from those causing the most severe cases of ALTB and are more similar in distribution to the viruses responsible for the pharyngitis-bronchitis syndrome. Yet, it should be noted that none of our severe ALTB cases yielded parainfluenza type 1 virus.

It is well-known that infection with parainfluenza 3 virus may occur in both children and adults [24] even in the presence of a moderate antibody level. In these cases, however, the resulting illness is relatively mild. The fact that parainfluenza viruses can be isolated more easily from primary infections of children than from adult cases is attributed by BLOOM *et al.* [33] to immunological factors. The fact that we have isolated viruses from children suffering from recurrent attacks at nearly the same rate as from primary cases might indicate that the virus causing the relapse was different from those responsible for earlier manifestations.

We suppose that a special allergic diathesis is a prerequisite of attacks of acute dyspnoea and stridor on 5 or 6 occasions within a short period of time. Such children develop the same syndrome irrespective of the virus causing the infection.

The incidence of neutralizing antibodies to parainfluenza 2 and 3 viruses agreed well with literary data [36]. Unfortunately, we had no parainfluenza 1 virus (HA2) at sufficient concentrations. Considering that according to CHANOCK *et al.* [4] and COOK *et al.* [34] the Sendai strain is closely related to the HA2 strain, we titrated the HI antibodies in human sera against Sendai virus. It should, however, be noted that ZHDANOV and BUKRINSKAYA [35] and LAPLACA and MOSCOVICI could not confirm the close relation between HA2 and Sendai viruses [36]. The results of our titrations, although they provided only an unsatisfactory, indirect, picture of the HA2 antibodies in our material, are suggestive of a considerable antigenic relation between parainfluenza type 3 and the Sendai virus.

As regards the relationship between mumps virus and parainfluenza viruses, examination of paired sera from older children may provide some information. The present results are consistent with those of STARK *et al.* [37].

The homologous antibody response of most of the children having yielded RS virus were found to be low, probably because the interval between the two samplings was too short.

Isolation of two viruses from the same patient and the demonstration of a simultaneous immune response to two viruses have already been reported by other investigators. In the material analysed by HILLEMAN *et al.* [38] the joint occurrence of RS virus and adenoviruses was the most common. These authors assume that RS virus may activate latent adenovirus infections. In our material simultaneous antibody responses to adenovirus + parainfluenza virus and adenovirus + RS virus were most often observed. Presumably these viruses can mutually activate each other. The fact that persistent carriership of parainfluenza viruses can be established in cell cultures, support this assumption.

Simultaneous antibody responses to parainfluenza 2 or 3 viruses and to influenza B (CF antibodies) as well as to parainfluenza 2 and 3 viruses were observed during relapses. Considering that cases similar in clinical picture but different in the aetiological factor are kept in the same ward, in such instances cross infections cannot be excluded. It is, however, also possible that the antibody response is not quite specific.

Isolation of RS and parainfluenza 2 virus in the course of the present study was the first isolation of these viruses in Hungary. Parainfluenza 1 and 3 viruses have already been isolated by KISZEL [39] and BÉLÁDI *et al.* [40, 41], respectively.

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INCIDENCE OF SV-40 VIRUS NEUTRALIZING ANTIBODIES IN SERA OF LABORATORY WORKERS

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Summary. The presence of SV-40 virus neutralizing antibodies was demonstrated in 18 (60 per cent) of a total of 30 persons handling rhesus monkeys or manipulating tissue cultures from the same animals.

The incidence of SV-40 antibodies was higher in persons vaccinated with two or more doses of Salk vaccine than in untreated persons.

The geometric mean titre (94) in laboratory workers was 23fold of the maximum value (4) in Salk-vaccinated controls.

The possible aerogenic spread of laboratory SV-40 infections is supposed and discussed.

Since the discovery of SV-40 by SWEET and HILLEMAN [14], many reports have been published on the incidence of this agent in monkeys and in tissue cultures prepared from the kidneys of these animals [9, 11, 14, 16, 17]. In our laboratory the incidence of SV-40 virus contamination was 49 per cent in tissue cultures charges prepared of pooled kidney trypsinizates of 4 to 6 monkeys each [7].

The high incidence of SV-40 contamination has been described [3, 5, 9, 10, 14] in both killed and live vaccines prepared from virus material obtained on monkey kidney tissue cultures. The interest in this agent has been remarkably enhanced by the discovery of its ability to produce malignant tumours in newborn hamsters [1, 2, 4, 15].

The present studies were performed with the aim to clarify whether infection with SV-40 may take place under laboratory conditions in persons handling monkeys or working with tissue cultures from kidneys of such animals. The infection of humans was detected by virus-neutralization test.

Materials and methods

Sera. Test sera were collected from laboratory workers handling monkeys or manipulating monkey kidney cultures. The number of persons exposed to direct or indirect contact with SV-40 virus totalled 30. Control sera were collected from a total of 32 persons entirely lacking contact with either monkeys or tissue cultures. Sera were inactivated for 30 minutes at 56°C and stored at 4°C until used. Serum dilutions were prepared in *Parker*'s 199 solution.

Tissue cultures. Tests were performed in primary kidney cultures of African vervet monkeys *(Cercopithecus aethiops)*. The growth medium consisted of *Hanks*' solution containing

0.5 per cent lactalbumin hydrolysate and 2 per cent calf serum. Cultures were ready for infection on the 7th or 8th day following their preparation. Infected cultures were maintained in *Parker*'s 199 solution with 6 to 8 per cent calf serum. The absence of any agents from the cultures was verified by the lack of cytopathic changes in the control cultures.

Virus. Strain A—426 of SV-40 virus was kindly supplied by M. P. CHUMAKOV (Institute of Poliomyelitis and Encephalitis Virus Research, Moscow). A standard stock of this virus has been prepared in our laboratory. The stock had a titre between 10^{-6} to 10^{-7}. It was distributed into 1 ml ampoules and stored at −20°C until used.

The actual titre of the virus was always determined parallel to the neutralization test.

Neutralization test. Appropriate serum dilutions in 0.2 ml were mixed with an equal volume of virus containing 100 to 200 CPD_{50}. Final dilutions of sera were 1 : 4, 1 : 16, 1 : 64, 1 : 256 and 1 : 1024. After thorough shaking, the mixtures were incubated for 2 hours at 37°C then at 4°C overnight and finally at room temperature for 30 minutes. The material was then inoculated into 3 tubes 0.1 ml each. After 30 minutes adsorption at room temperature, 0.9 ml of maintenance medium was added to each tube and the inoculated cultures were incubated at 37°C. Final reading was taken on the 21st day of incubation. The serum dilution protecting at least 2 out of the 3 cultures inoculated was considered positive.

Results

Results are summarized in Table I. The SV-40 antibody content of a total of 62 sera was determined. Of these, 21 yielded positive results in the neutralization test. The probability of contact and the incidence of neutralizing antibodies showed the following relations. Out of the 7 persons handling only monkeys (group 1), 3 had antibodies. Group 2 included 15 persons manipulating only monkey kidney cell cultures. Nine of them had antibodies.

Table I

SV-40 virus neutralizing antibodies in the sera of laboratory workers

Group	Vaccination with Salk vaccine in the period 1957—59		Total	Per cent of positives
	≥ 2 doses	Not vaccinated		
1	1/1*	2/6	3/7	
2	8/12	1/3	9/15	
3	4/6	2/2	6/8	
1—3 Total	13/19	5/11	18/30	60
4	3/14	0/18	3/32	9.3

Remarks :

1. Persons, working regularly with monkeys or visiting the monkey house.
2. Persons, working with rhesus monkey kidney tissue cultures only.
3. Persons, working with rhesus monkey tissue cultures and irregularly visiting the monkey house.
4. Control = Persons who had never worked with tissue cultures and negated the possibility of contact with rhesus monkeys.

* Nominator = Number of persons with SV-40 antibodies. Denumerator = Number of persons tested.

Table II

Titres of SV-40 neutralizing antibodies in the sera of persons of different groups

Groups**	Number of persons with titre				
	1 : 4	1 : 16	1 : 64	1 : 256	1 : 1024
1	2	—	—	1	—
2	3	3	2	—	1
3	1	2	3	—	—
Control	3	—	—	—	—

** See Table I.

Out of the 8 persons having had frequent contact with both monkeys and monkey kidney cultures (group 3), 6 were found to possess neutralizing antibodies against SV-40. Thus for this group the total incidence of positive serology was 60 per cent (18 out of 30), whereas in the control group the incidence was only 9.3 per cent (3 out of 32). This difference appeared to be remarkable enough to draw attention to the possibility of laboratory infections with SV-40 virus of persons handling rhesus monkeys or primary tissue cultures from the same animals.

Results were then evaluated with respect to preceding Salk vaccinations in the period 1957—1959. In the control group all the 3 persons with positive neutralization test belonged to the 14 individuals given two or more doses of Salk vaccine at an earlier period. Thus 21 per cent of Salk-vaccinated control persons (3 out of 14) had SV-40 antibodies. In the test group a total of 19 persons had been immunized with SALK's vaccine. Of these 13 (68 per cent) yielded positive serological tests. None of the unvaccinated controls had SV-40 antibodies. Among the unvaccinated 11 laboratory workers 5 (45 per cent) exhibited positive neutralization tests with SV-40 virus.

There was a complete accordance between the titres and the results presented in Table I (see Table II). No control serum was found to contain neutralizing antibodies in dilutions higher than 1 : 4, while most laboratory workers had high antibody titres.

Discussion

The data presented are in agreement with those published by CHUMAKOVA [18] and POLNA [13] on the incidence of SV-40 antibodies in sera of persons manipulating kidney tissue cultures of rhesus monkeys. In addition to these data the incidence of SV-40 antibodies in (17) full-time tissue culture workers has been calculated; 13 (76 per cent) among them had antibodies. This figure correlates well with the 86 per cent obtained by CHUMAKOVA [18].

The geometric mean titre of SV-40 antibodies was 94 in the laboratory workers. This value was 23fold of that found in the control group. POLNA [13] gives no titre values, whereas CHUMAKOVA reported titres from 1 : 256 to 1 : 1024 and higher than 1 : 1024 in 30 and 10 cases, respectively. These values remarkably exceeded those found by us. A possible explanation of this phenomenon may be the fact that in our laboratory tissue culture work has been conducted on a lesser scale than in CHUMAKOVA's laboratories. The differences in antibody levels found in the two institutes and the possible causes of the phenomenon may be responsible for the lack of correlation between Salk vaccination and the incidence of SV-40 antibodies in CHUMAKOVA's material. Such a correlation was, however, clearly demonstrable in the group studied by us. SV-40 antibodies were present in 68 per cent vaccinated and in 45 per cent unvaccinated laboratory workers. The difference of 23 per cent agrees well with the figure obtained for the incidence of SV-40 antibodies (21 per cent) in the Salk vaccinees of the control group. Theoretically one would expect a significant difference between these two figures. It appears that in cases where the small amount of SV-40 antigen present in the Salk vaccine was insufficient to produce measurable antibody production, repeated laboratory infections might have served as challenges enhancing the immune response. These challenges were certainly lacking in the control group. The experimental checking of this supposition is outside the scope of the present study.

As to the possible mechanisms of laboratory infections with SV-40, we have to content ourselves with theoretical considerations. It has repeatedly been demonstrated that, in relation to Salk vaccination, the parenteral administration of SV-40 antigen results in antibody formation [5, 8, 14, 18]. Parenteral infections are rare, though not impossible in persons handling monkeys or manipulating tissue cultures (injury of hands). Enteral infection is also possible. Evidence is however, lacking that such an infection would result in antibody production. In fact, we could not detect SV-40 antibodies in the sera of 145 children 9 to 12 months of age fed with live polio vaccine carrying SV-40 contamination. The latter virus was, however, repeatedly isolated from the faeces of vaccinated individuals [6]. The immunogenic effect of nasally administered SV-40 virus has been demonstrated on volunteers by MORRIS *et al.* [12], who observed antibody formation in 22 of 35 adults infected nasally with 10^4 CPD_{50} of SV-40 virus. Under laboratory conditions the chances for nasal infection exceed remarkably those of either enteral or parenteral infections. The laboratory results obtained up to now appeared to support this view. Thus we have to suppose that the antibodies revealed by our studies had resulted from nasal infection of the laboratory workers. However the possibility of parenteral infection cannot be excluded, either.

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HAEMAGGLUTINATION-INHIBITING ANTIBODIES TO ADENOVIRUS TYPES 8, 9 AND 10 IN PAIRED SERA OF PATIENTS WITH EPIDEMIC KERATOCONJUNCTIVITIS

By

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(Received February 1, 1965)

Summary. During an outbreak of epidemic keratoconjunctivitis caused by adenovirus type 8, the HI antibodies to adenovirus types 8, 9 and 10 were titrated in 122 serum pairs. In accordance with literary data, most of the convalescent sera contained antibodies to types 9 and 10, in addition to the homologous antibodies.

Of the hyperimmune rabbit sera those prepared to types 8 and 9 gave cross reaction in the HI test, whereas the type 8 and type 9 rabbit sera gave no HI reaction with type 10 virus, and *vice versa*.

The HI serum antibodies to adenovirus types 8, 9 and 10 were still demonstrable two years after convalescence from EKC.

ROSEN [1], who was the first to report on haemagglutination by adenoviruses, evolved the haemagglutination-inhibition (HI) test suitable for typing adenoviruses [1, 2]. When the test was applied in a wide range it was found that some of the adenovirus serotypes give cross reactions, thus infections by certain types are followed by heterotypic HI antibody responses [3—7].

In Hungary, NÁSZ *et al.* [8] have demonstrated HI antibodies to types 9 and 10 in the sera of EKC convalescents, in addition to the homologous type 8 antibodies. In the present report similar observations are discussed. The paired sera under study were collected during the wide outbreak of EKC in Budapest, 1961—1962.

Materials and methods

Virus strains. The prototype strains of adenovirus types 8, 9 and 10 had originated from the collection of H. G. PEREIRA (London). We received them by the courtesy of DR. I. BÉLÁDI (Szeged). The viral suspensions were obtained from HeLa cell cultures as published earlier [9].

Hyperimmune typing sera. These were prepared in rabbits according to DÖMÖK *et al.* [10].

Serum samples. Paired sera were collected during the Budapest EKC outbreak in 1961—1962. The acute-phase specimens were taken in the first week of illness, the convalescent-phase sera between the 15th and 60th days after onset. From each of 11 subjects a third sample was obtained two years later. The sera of these patients were comparatively tested for HI and neutralizing antibodies.

Neutralization tests. Twofold dilution series of sera were tested against about 20 CPD_{50} of virus. The first dilution was usually 1 : 4, in certain cases, when serum was not available in sufficient amounts, 1 : 8 or 1 : 20. Each serum-virus mixture was inoculated into three tube cultures of HeLa cells. The titres were calculated from reading on the seventh day of incubation.

HI tests were performed as published elsewhere [9], except that human 0 erythrocytes were used. Before titration the sera were absorbed with kaolin.

Results

HI antibodies to adenovirus types 8, 9 and 10 were titrated in 122 serum pairs of patients with EKC. The results are summarized in Table I. It is seen that about two-third of the acute-phase sera proved to be negative to each of types 8, 9 and 10. In view of the low number of sera, the positive correlation among the titres to different types is not quite convincing.

The correlations are, however, convincing for the convalescent sera, particularly for types 8 and 9. To both of these types 78 convalescent sera gave equal titres. Nevertheless, in a few serum samples the titres to these two types were definitely divergent, *e.g.* 3 sera had no antibodies detectable in 1 : 10 dilution to type 9, whereas the same sera showed titres of 1 : 80 to adeno-

Table I

Distribution of 122 cases of EKC by serum HI titres to adenovirus types 8, 9 and 10

(a) Type 8 versus type 9

		Acute-phase sera					Convalescent-phase sera				
		Titre to type 9			Total	$\bar{x}g$ for type 9	Titre to type 9			Total	$\bar{x}g$ for type 9
		<10	10—40	≧80			<10	10—40	≧80		
Titre to type 8	<10	66	16	1	83	6	38	9	3	50	7
	10—40	13	14	2	29	13	10	20	8	38	21
	≧80	1	3	6	10	56	3	11	20	34	59
Total		80	33	9	122		51	40	31	122	
$\bar{x}g$ for type 8		6	11	46			8	21	69		

(b) Type 8 versus type 10

		Acute-phase sera					Convalescent-phase sera				
		Titre to type 10			Total	$\bar{x}g$ for type 10	Titre to type 10			Total	$\bar{x}g$ for type 10
		<10	10—40	≧80			<10	10—40	≧80		
Titre to type 8	<10	61	22	—	83	7	36	13	1	50	7
	10—40	14	14	1	29	11	14	19	5	38	12
	≧80	3	4	3	10	23	7	12	15	34	26
Total		78	40	4	122		57	44	21	122	
$\bar{x}g$ for type 8		7	9	56			10	20	112		

(c) Type 9 versus type 10

		Acute-phase sera					Convalescent-phase sera				
		Titre to type 10			Total	$\bar{x}g$ for type 10	Titre to type 10			Total	$\bar{x}g$ for type 10
		<10	10—40	≧80			<10	10—40	≧80		
Titre to type 9	<10	64	15	1	80	6	42	9	—	51	6
	10—40	10	21	2	33	14	10	25	5	40	15
	≧80	4	4	1	9	14	5	10	16	31	37
Total		78	40	4	122		57	44	21	122	
$\bar{x}g$ for type 9		7	15	24			8	29	129		

$\bar{x}g$ = geometric mean

virus type 8. Conversely, a serum having no detectable antibodies to type 8 had a titre $\geq$ 1 : 320 to type 9. There was a correlation between the anti-type 8 and anti-type 10 titres as well as the anti-type 9 and anti-type 10 titres. These correlations were, however, less pregnant, as shown in Table I. There were few convalescent sera with a higher titre to type 10 than to type 8 or 9 of virus; well-defined deviations in the opposite sense were considerably more frequent.

As shown in Table II, a rise in titre to type 8, 9 and 10 was demonstrated in 62, 52 and 45 cases, respectively; the rise was at least fourfold in 46, 37 and 25 cases, respectively. Though the differences were not too marked, it is obvious that the causative agent of the epidemic, *viz.* adenovirus type 8, was the serotype to which a rise in antibody titre was demonstrable most frequently. Type 8 was followed by type 9, and antibody response to type 10 was the least frequent.

Table III shows the cross neutralization and HI titres of our typing sera (rabbit sera). There was a complete cross inhibition between type 8 immune serum and the type 9 strain, whereas the homologous HI titre of the anti-type 9 serum was four times higher than its titre to the type 8 virus. There was no detectable cross inhibition between adenovirus type 10 on the one hand and types 8 and 9 on the other.

We were not able to demonstrate cross reactions in the neutralization test. Considering that the homologous titre of the sera was only 2—4 times higher than the lowest dilution in which the sera were tested, the existence of a cross reaction detectable by a more sensitive technique could not be excluded.

From each of 11 cases examined during the epidemic period in 1961—1962, one or two additional serum samples were obtained during the 2-year period

Table II

Acute- and convalescent-phase HI titres of paired sera of 122 EKC patients

Convalescent-phase titres	Adeno 8							Adeno 9							Adeno 10						
	≤10	10	20	40	80	160	≥320	<10	10	20	40	80	160	≥320	<10	10	20	40	80	160	≥320
≥320	3				1	2		4	2	2			2	1			1				
160	4	1		2	1			4	2		3	1	1		1	2	1		1	1	
80	7	2	5	3	2		1	3	1	1	1	2	1		4	2	5	2	1		
40	9	1	1	1	1			6	2	3	3				2	2	2	2			
20	10	1	3					7	1	6	1				5	1	5	2	1		
10	8	1	1	1				7	2	1	1				14	7	1				
<10	42	5	1		1	1		49	1			1			52	4	1				

Acute-phase titres

Table III

Cross HI and neutralization reactions in rabbit hyperimmune sera

Rabbit serum type	Neutralization titre* to 10 CPD_{50} adenovirus type			HI titre* to 4 HA units of adenovirus type		
	8	9	10	8	9	10
Adenovirus 8	50	<25	<25	640	640	<10
Adenovirus 9	<25	100	<25	320	1280	<10
Adenovirus 10	<25	<25	100	<10	<10	2560

* Reciprocals

Table IV

CF and HI titres in the sera of EKC patients in the acute-phase, convalescent-phase, and two years thereafter

Case No.	Time after onset	HI titres* to adenovirus			CF
		type 8	type 9	type 10	
1.	1 day	—	—	—	4
	21 days	—	—	—	4
	77 days	20	—	—	.
	2 years	10	—	10	<4
2.	6 days	20	.	.	<4
	20 days	10	—	—	16
	34 days	10	10	20	16
	2 years	20	10	10	8
3.	4 days	—	—	10	<4
	17 days	20	—	10	<4
	2 years	40	10	20	<4
4.	3 days	—	—	—	16
	25 days	40	.	.	16
	41 days	80 (16)	10	—	.
	2 years	40	20	40	16
5.	2 days	—	—	—	4
	14 days	20	40	10	4
	19 days	40	40	20	16
	2 years	20	20	20	16
6.	3 days	—	—	—	16
	13 days	20	—	—	16
	26 days	10	—	10	16
	2 years	20	—	10 (32)	8
7.	2 days	40	—	—	16
	14 days	80	80	80	32
	27 days	160	80	80	64
	2 years	40	80	80	4
8.	3 days	10	—	—	<4
	17 days	80	40	20	32
	30 days	80	.	.	32
	2 years	20	20	20	<4
9.	3 days	—	—	—	4
	12 days	40	—	—	16
	42 days	40 (16)	—	—	16
	2 years	40	10	20	8
10.	2 days	—	—	20	<4
	8 days	80	20	80	<4
	2 years	40	40	160 (16)	<4
11.	3 days	—	—	—	<4
	32 days	20	—	—	8
	43 days	20	10	10	.
	2 years	20	10	10	<4

. = not tested
— = <10
* = reciprocals

Figures in parantheses mean neutralization titre. The other tests showed no neutralizing antibody at 1 : 16 dilution

following convalescence. The complement-fixing (CF), HI and neutralization titres of these sera were tested against adenovirus types 8, 9 and 10 (Table IV).

The convalescent titres decreased only slightly, and never disappeared during the two-year period. In several cases HI antibody could be demonstrated after two years to such types to which HI antibody could not be detected in the convalescent sample.

The neutralization titres were low. There were only two cases where the titre to adenovirus type 8 attained the 1 : 16 level during convalescence. Higher titres were not observed. Two years after convalescence we failed to detect antibodies even by examining 1 : 4 diluted sera. In two cases, however, the sample taken two years after convalescence elicited the highest titres (1 : 16 and 1 : 32) to adenovirus type 10. (The samples taken at different times were titrated simultaneously, under the same circumstances). In the other sera, irrespective of type, the antibody titre did not attain the 1 : 16 level.

Discussion

On the basis of HI tests carried out with rabbit hyperimmune sera [2, 6, 8] a serological cross reaction was obviously detectable between adenovirus types 8 and 9. This cross reaction was well-defined in our rabbit sera as well. On the other hand, the occurrence of cross reactions with type 10 hyperimmune sera is still uncertain. Our results are consistent with those of SEVER *et al.* [6] and RAFAJKO [11] inasmuch that we were unable to establish a cross reaction between adenovirus types 8 and 9 on the one hand and type 10 on the other.

We have confirmed the appearance of HI antibodies to adenovirus types 9 and 10 in the serum of EKC convalescents. There exists a well-defined correlation between the type 8 and type 9 antibody titres and a less pronounced correlation with the type 10 titres. The parallelism manifested itself also with the rise in titre during illness.

In spite of the cross reactions, the HI test should be considered suitable for revealing the pathogenic agent of EKC cases, as except for a single publication [12] there is no basis for assuming any role to types 9 and 10 in the aetiology of EKC-like cases. To types 3, 7 and 14, which have been suspected to have an aetiological role in cases similar to EKC, EKC convalescents produce no HI antibodies when the illness is due to adenovirus type 8 [9].

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ANTIVIRAL ACTIVITY OF TWO AMINO-ETHYL-GUANIDINE AND AN EUGENOL DERIVATIVES

By

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(Received March 15, 1965)

Summary. Two amino-ethyl-guanidine and an eugenol derivatives were tested for antiviral activity in primary monkey-kidney cell cultures, using the plaque technique. The eugenol derivative moderately, the two guanidine derivatives (Quanethidin, PYG) definitely, inhibited the plaque formation by the RNA viruses poliovirus LSc 2ab, coxsackievirus B-3 (prototype strain) and the DNA virus *Herpesvirus hominis*. The effect of PYG was particularly remarkable; this compound inhibited plaque formation by each of the above viruses at a concentration of 10 μg/ml. The effect of PYG and Quanethidine cannot be attributed to their guanidine component exclusively.

Most recently the number of works dealing with antiviral compounds has considerably increased. It has been shown that compounds highly different in chemical structure may inhibit viral production. Thus, 2-(α-hydroxybenzyl)-benzimidazol [1—3] as well as guanidine [4] inhibit reproduction of some enteroviruses (RNA viruses) whereas *Herpesvirus hominis* (DNA virus) is inhibited by 5-iodo-2-deoxyuridine [5]. 3-iodo-2-methylmercapto-4-thio-6-azauracyl inhibits the reproduction of both RNA and DNA viruses [6, 7]. Although an excessive number of compounds have been tested for antiviral activity, only a few of them have been put on the market [8, 9].

In the present study the virostatic activity of two guanidine alkylamines and an eugenol alkylamine has been examined.

Materials and methods

Cell culture. Primary monkey-kidney monolayers were established in test tubes, and in Petri dishes 9 cm or 5 cm in diameter.

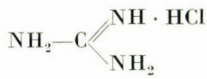
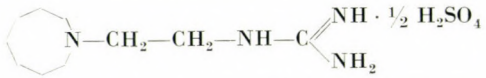
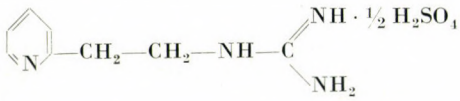
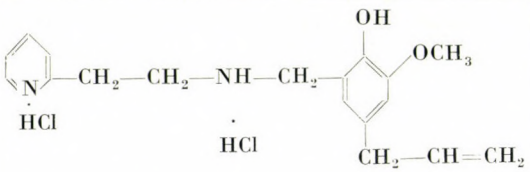
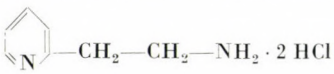
Viruses. The wild Mahoney and the attenuated LSc 2ab strains, both representing poliovirus type 1, the prototype strains of echovirus type 19, and coxsackievirus B3, and a herpes simplex virus strain [11] were used. The enterovirus strains were kindly supplied by DR. I. DÖMÖK (State Institute of Hygiene, Budapest).

Chemicals. 2-(β-aminoaethyl)pyridine (AEP), 2-(pyridylethyl)-guanidine sulphate (PYG) [12], 2-(5-allyl-3-methoxy-2-hydroxybenzyl)-β-(α-pyridyl)-ethyl amine (H-99) [13], and Quanethidine (2-(-octahydro-1-azocynyl)ethyl guanidine sulphate) [12] were tested for antiviral activity. The well-known antiviral compound guanidine HCl served as control. Chemical structure and molecular weight of the compounds are given in Table I.

Plaque method. The method of HSIUNG and MELNICK [14] was applied with some modification. The chemicals were incorporated in the semisolid medium at different concentrations (10, 20, 50 or 100 μg per ml). When a compound was applied late after infection, it was incorporated in a second nutrient layer. Viral suspensions were diluted to contain 100 plaque-forming units (PFU) in 0.2 ml (the inoculated volume). In every case three parallel titrations were made. Evaluation took place on the fourth day after inoculation.

Table I

Data of compounds used in the experiments

Substance	Structure	Molecular weight
Guanidine HCl		95.54
Quanethidine (2-(-octahydro-1-azocynil)ethyl-guanidine sulphate)		247.36
PYG (2-(pyridylethyl)-guanidine sulphate)		213.36
H-99 (2-(5-allyl-3-methoxy-2-hydroxybenzyl)-β-(α-pyridyl)ethyl guanidine sulphate)		371.32
AEP (2-(β-aminoethyl) pyridine)		195.13

Results

Effects of Quanethidine, PYG, H-99 and AEP on plaque formation by the LSc 2ab strain. A hundred μg/ml was incorporated in the nutrient layer, which was then poured on top of the monolayer 1 hour after infection.

Table II

Inhibition of plaque forming capacity of LSc virus by PYG, Quanethidine, H-99 and AEP

Compound	Concentration μg/ml	Number of plaques
—	—	130
Quanethidine	100	0
PYG	100	0
H-99	100	0
—	—	95
AEP	100	80

As shown in Table II, plaque formation by LSc 2ab virus was inhibited by both guanidine derivatives and the eugenol derivative. It was practically not inhibited by AEP.

Effect of Quanethidine and PYG on plaque formation by the LSc 2ab strain when antiviral agent was added at different periods after viral infection. As Table III shows, both of these compounds inhibited plaque formation, even when added after a relatively long period following viral infection. PYG exceeded Quanethidine in inhibition; the former compound prevented the formation of visible plaques even when added 32 hours after inoculation.

Table III

Virus inhibitory effect of Quanethidine and PYG applied at different times after virus infection

Compound	Concentration μg/ml	Time of addition after infection, hours	Number of plaques
—	—	—	98
Quanethidine	100	4	0
		24	45
PYG	100	4	0
		24	0
		32	12

Table IV

Inhibition of plaque formation of LSc virus by di ferent compounds

Compound	Concentration μg/ml	Number of plaques
—	—	55
Quanethidine	20	0
PYG	20	0
Guanidine	20	8
H-99	20	48

The effect of 20 μg/ml concentrations of Quanethidine, PYG, H-99 and guanidine on plaque formation by the LSc 2ab strain. Table IV shows that both guanidine derivatives (Quanethidine and PYG) as well as guanidine HCl serving as control markedly inhibited the plaque formation when 20 μg/ml concentrations were added 1 hour after inoculation. The eugenol derivative (H-99) was ineffective at that concentration.

The two compounds (Quanethidine and PYG) that had proved most effective in the above experiments, were tested against some further viruses.

Effect of PYG and Quanethidine on plaque formation by other virus strains. Table V shows how PYG and Quanethidine inhibited plaque formation by the Mahoney strain and the Mahoney, echovirus type 19, coxsackievirus type B3 and the herpesvirus strains, respectively, when the antiviral agent was applied at a concentration of 100 μg/ml.

Table V

Virus inhibitory effect of Quanethidine and PYG on different virus strains

Compound	Concentration μg/ml	Virus strain	Number of plaques
—	—	Mahoney	53
Quanethidine	100	Mahoney	0
PYG	100	Mahoney	0
—	—	Echovirus 19	75
Quanethidine	100	Echovirus 19	0
—	—	Coxsackievirus B3	25
Quanethidine	100	Coxsackievirus B3	0
—	—	Herpesvirus	105
Quanethidine	100	Herpesvirus	0

Not only the representatives of RNA viruses (LSc, echovirus 19, coxsackievirus B3) but even a DNA virus (herpesvirus) was inhibited by Quanethidine. Based on these results we assumed a similar effect for PYG, which had been tested only against the Mahoney virus. As each of these two com-

Table VI

Effect of Quanethidine and PYG on plaque formation by different virus strains

Compound	Concentration μg/ml	Virus strain	Number of plaques
—	—	LSc 2ab	76
Quanethidine	10	LSc 2ab	52
PYG	10	LSc 2ab	8
—	—	Echovirus 19	45
Quanethidine	10	Echovirus 19	50
PYG	10	Echovirus 19	28
—	—	Coxsackievirus B3	30
Quanethidine	10	Coxsackievirus B3	8
PYG	10	Coxsackievirus B3	0
—	—	Herpesvirus	65
Quanethidine	10	Herpesvirus	60
PYG	10	Herpesvirus	12

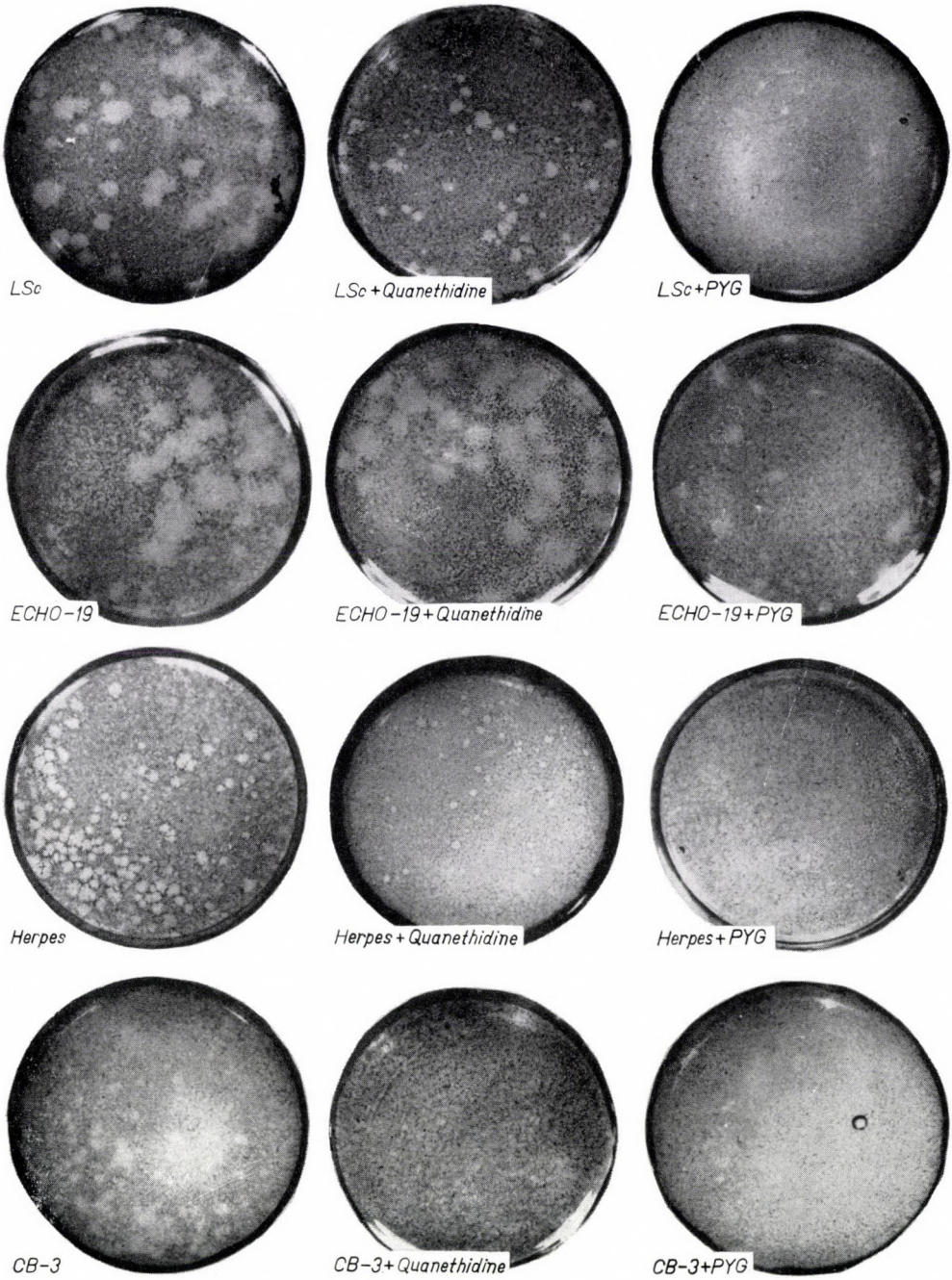


Fig. 1. Inhibitory effect of Quanethidine (10 μg/ml) and of PYG on different virus strains

pounds proved to be effective when the nutrient layer contained 20 μg/ml, it was reasonable to examine their effect at a concentration of 10 μg/ml. The corresponding data are shown in Table VI.

It is clearly seen that the strains, except the echovirus type 19 strain, were inhibited to about the same degree by the two compounds. According to the number of plaques, inhibition was poor in several cases, but, as shown in Fig. 1, the size of the plaques was also reduced in the presence of 10 μg/ml of the antiviral agent.

Echovirus type 19 was not influenced by Quanethidine.

Mechanism of action of PYG. In the above experiments, PYG was the most active antiviral agent. This compound was therefore examined for the mechanism of action.

To examine the effect of PYG *in vitro*, LSc 2ab virus was mixed with PYG to make a mixture containing 100 PFU of virus and 100 μg of PYG per 0.2 ml. This was incubated at 37°C for 3 hours and, subsequently, added to monolayers. Control cultures were inoculated with virus without PYG. After a 30 minute adsorption period the cultures were washed with PBS three times and covered with a nutrient layer. There was no difference in plaque count between the control plate and that inoculated with PYG-treated virus, indicating that PYG exerted no effect on the LSc 2ab virions.

Adsorption of LSc 2ab virus to cells was not influenced by PYG, as proved by the following experiment.

Monolayers were infected with 100 PFU of LSc 2ab virus in the presence of 200 μg/ml, or without, PYG. After 2-hours adsorption the monolayers were washed several times with PBS and subsequently covered with a nutrient layer. There was no significant difference in plaque count between the control plates and those with PYG.

The mechanism of action of PYG cannot be identical with that of guanidine, for the former is more effective not only in reducing plaque count, but also in its wider virus spectrum; even herpesvirus, a representative of DNA

Table VII

Comparison of virus inhibitory effect of PYG and guanidine on RNA (LSc) and DNA (Herpes) viruses

Compound	Concentration μg/ml	Virus strain	Number of plaques
—	—	LSc 2ab	45
Guanidine	10	LSc 2ab	12
PYG	10	LSc 2ab	3
—	—	Herpesvirus	98
Guanidine	10	Herpesvirus	102
PYG	10	Herpesvirus	15

viruses, was inhibited. A comparison of the effects of the two compounds is given in Table VII.

The effect of PYG in different phases of the reproduction cycle of the LSc 2ab strain was also studied. Preliminary data suggest that PYG exerts its effect in the 3rd and 4th hours after infection.

Discussion

Among the antiviral compounds under study AEP proved to be ineffective, whereas the eugenol derivative H-99 was moderately active in inhibiting the reproduction of the LSc 2ab strain. Yet, we think that examination of further eugenol derivatives for antiviral effect would be reasonable. The two guanidine derivatives PYG and Quanethidine surpassed guanidine both in degree of inhibition and virus spectrum. PYG was the most effective, inhibiting the reproduction of both the RNA viruses LSc 2ab, echovirus type 19 and coxsackievirus B3 and the DNA virus *Herpesvirus hominis*. In similar experiments AEP, which is incorporated in the PYG molecule, was ineffective. Nevertheless, the corresponding radical in the PYG molecule must be of importance, considering that its coupling to guanidine increases the effect and widens the virus spectrum of the latter.

It should be noted that both PYG and Quanethidine are known to reduce blood pressure; Quanethidine has even been used as an antihypertensive drug [15].

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UNTERSUCHUNG DER KATALASEAKTIVITÄTS-ÄNDERUNG VON FUCIDIN-RESISTENTEN STAPHYLOCOCCUS AUREUS-KULTUREN

Von

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(Eingegangen am 12 April 1965)

Zusammenfassung. Im Laufe der Fucidinadaptation von Staphylococcus aureus-Kulturen hört die Katalaseaktivität der Zellen allmählich auf. Bei der fucidinfesten Kultur kommt es nicht zur Enzyminduktion, weil sich das Gleichgewicht der Prozesse während der Adaptation in Richtung katalaseunabhängiger Oxydationsmechanismen verschiebt und deshalb das Fucidin die Atmungsintensität der adaptierten Mikroorganismen nicht beeinflußt.

Untersuchungen über die Antibiotikumempfindlichkeit von Staphylokokkusstämmen haben ergeben, daß die Toleranz der Bakterien steigenden Antibiotikumkonzentrationen gegenüber allmählich zunimmt und diese Adaptation später in eine Resistenz übergehen kann. Eine andere Entstehungsart der Resistenz ist, daß genetisch mutante, primär resistente Keime selektiert werden [1]. Im Laufe der Adaptation sind die Stoffwechseländerungen des Mikroorganismus an Hand des Studiums gewisser Enzymsysteme gut verfolgbar, weil diese Veränderungen sowohl durch die gesteigerte Produktion der gehemmten Enzyme als auch durch die erhöhte Einbeziehung der schon vorhandenen, aber weniger in Anspruch genommenen Reaktionswege zustande kommen können.

Beim Studium der antibakteriellen Wirkung des über ein Sterangerüst verfügenden Fucidins [2] ist nachgewiesen worden, daß die meisten Staphylokokkenstämme ihm gegenüber sensitiv sind [3]. Diese Stämme verfügen über eine für die aeroben Mikroorganismen charakteristische Katalaseaktivität. Sofern das Fucidin den Oxydationsmechanismus beeinflußt, ist anzunehmen, daß die Antibiotikumwirkung auch die Katalaseaktivität nicht unberührt läßt.

Die Katalase ist eines jener induzierbaren Enzyme, welche in Gegenwart von Sauerstoff oder unter dem Einfluß des durch Reduktion im Zellstoffwechsel entstandenen Sauerstoffs gebildeten Peroxyds entstehen [4]. Was die Lokalisation der Katalase anbetrifft, so ist sie in etwa gleichen Mengen im Zellplasma und in den darin enthaltenen strukturellen Elementen (Mitochondrien, Mikrosomen, Kerne) verteilt [5, 6, 7], nach KAPLAN ist sie in erster Linie in komplexer Form an Ribonukleoproteide gebunden [8]. In Anbetracht der Funktion der Ribonukleinsäure [9] dürfte die Aktivität des Enzyms in engem Zusammenhang mit dem Zellwachstum stehen.

Material und Methoden

Bei dem in den Versuchen benutzten *Staphylococcus aureus*-Stamm handelte es sich um einen ziemlich verbreiteten Krankenhausstamm, der vorwiegend aus pemphigoiden Prozessen isoliert wird. Von den Antibiotika ist der Stamm gegenüber Chloramphenicol, Neomycin, Polymyxin B und Erythromycin empfindlich. Eine Typisierung mit den Grund-Phagen war nicht möglich und eine Lyse nur mit dem letztlich isolierten Phagen $42/D_1$ herbeizuführen. Der Stamm fermentiert Mannit und produziert Hämolysin, Koagulase und Phosphatase.

Zur Herstellung von Bouillon wurde pro Liter 8 g konzentrierter Fleischextrakt, 4 g Hefeextrakt (100 g Hefe in 100 g Wasser bei 1 Atü. 10 Minuten gekocht und filtriert), 1,6 g NaCl, 1,6 g Na_2HPO_4 und 2,4 g Pepton verwendet und das pH mit KOH oder H_3PO_4 auf 7,0 eingestellt.

Zur Züchtung wurden 40 ml Nährboden in 100 ml Erlenmeyer-Kolben gegeben und mit 2 ml von 16 Stunden alten Vorkulturen beimpft. Die Bebrütung erfolgte bei 37°C und einer Schüttelfrequenz von 100/min.

Die Angewöhnung der Mikroorganismen an Fucidin geschah allmählich durch serienmäßiges Überimpfen 10 Tage hindurch in Nährböden mit steigendem Fucidingehalt.

Der aus einem 5 μg/ml Fucidin enthaltenden Nährboden gezüchtete Stamm erwies sich auch größeren Antibiotikumdosen gegenüber als resistent.

Die Katalaseaktivität der zu verschiedenen Zeiten entnommenen Proben wurde jodometrisch [10] bestimmt und die erhaltenen Werte auf Startpunktaktivität umgerechnet [11].

Die Zellatmung, d. h. die Änderung des partialen Sauerstoffdruckes im Medium wurde mittels Potentialmessung [12] verfolgt, wobei einer gesättigten KCl-Kalomelelektrode eine glatte Platinelektrode gegenüber geschaltet wurde. Die Elektroden waren so gewählt, daß ihr Potential in destilliertem Wasser im Stickstoffstrom in gleicher Weise abnahm.

Ergebnisse

Die potentiometrische Verfolgung der Atmungsintensität der *Staph. aureus*-Kulturen [13] zeigte, daß die Verringerung der Potentialwerte während der logarithmischen Wachstumsphase am größten ist. Die Auswertung der Potentialkurvenänderung und der O_2-Tension [14] weist darauf hin, daß der intensivste Verbrauch des im Nährboden gelösten Sauerstoffbestandes nicht in den Bereich der niedrigen, sondern in den der höheren Potentialwerte fällt. Hier ist nämlich der Gehalt an gelöstem Sauerstoff noch hoch und die Möglichkeit seines Verbrauches durch die Zellen noch größer. Bei niedrigeren E_h-Werten ist bei wesentlich geringerer O_2-Konsumption eine größere Potentialveränderung zu beobachten, weil die Verminderung des Potentials der Verringerung des partialen O_2-Druckes logarithmisch proportional ist.

In Anbetracht dessen, daß der anfangs relativ hohe O_2-Gehalt von der lebenden Zelle allmählich verbraucht wird, kann aus der Potentialveränderung auf die Atmung der Zelle bzw. deren Intensität geschlossen werden. In den Fucidin-sensitiven Kulturen des *Staph. aureus* wird das im Laufe der Entwicklung gemessene Potential (Abb. 1, Kurve 1/b) in der logarithmischen Phase stark herabgesetzt und das Milieu nach Erschöpfung seines O_2-Bestandes immer mehr anaerob. Bei so niedriger Sauerstofftension hört die Zellvermehrung auf.

Der Fucidin-sensitive *Staph. aureus*-Stamm vermag sich bei dem gegebenen Fucidin-Gehalt infolge Aufhebung der aeroben Prozesse nicht zu vermehren, und so wird im E_h-Wert keine wesentliche Veränderung wahrnehmbar (Abb. 1, Kurve 1/a). Seine anfängliche geringgradige Verminderung ist

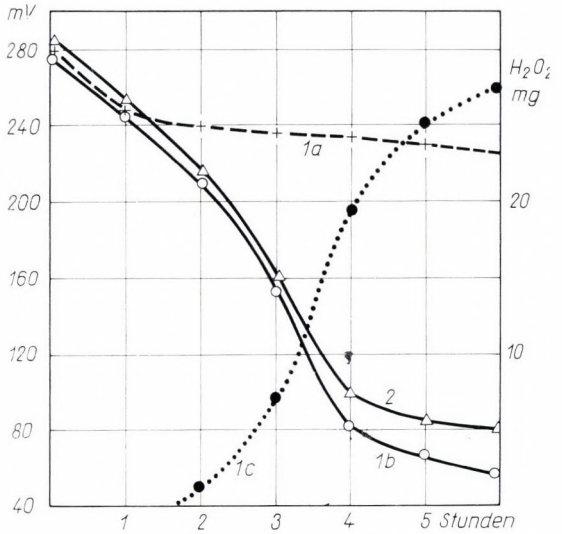

Abb. 1. Zusammenhang zwischen dem Potential der gegen Fucidin resistent gewordenen *Staphylococcus aureus*-Kultur (2), sowie dem Potential der fucidinfreien (1/b), der 5 μg/ml Fucidin enthaltenden Nährlösung (1/a) und der Katalaseaktivitätsänderung der der empfindlichen Kultur (1/c)

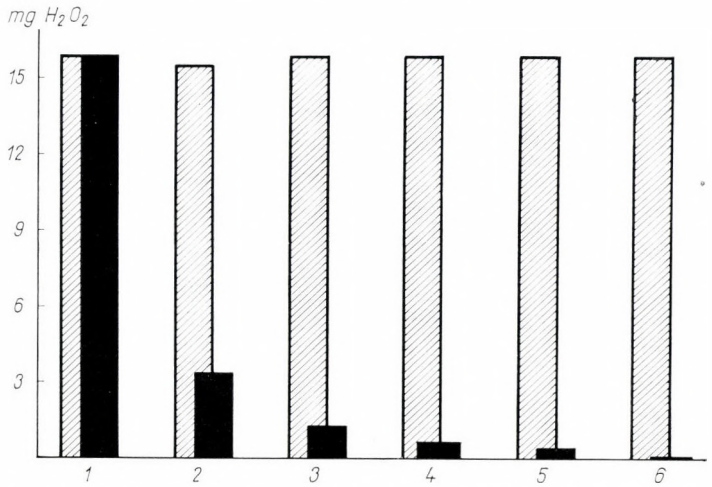

Abb. 2. Während der Erlangung der Fucidinresistenz des *Staphylococcus aureus* beobachtete Katalaseaktivitätsverminderung (schwarze Kolumnen) verglichen mit der Katalaseaktivität der empfindlichen Kultur (gestrichelte Kolumnen) bei der Züchtung in fucidinfreiem (1), bzw. 0,1 (2), 0,2 (3), 0,5 (4), 1 (5) und 5 μg/ml Fucidin-haltigem Nährboden (6)

die Folge des initialen O_2-Verbrauches des Inokulums. Die Zellatmung der Fucidin-resistenten Kultur war ähnlich der der sensitiven (Abb. 1, Kurve 2). Die Potentialänderung deutet darauf hin, daß die O_2-Konsumption der sensiblen und der resistenten Zellen als gleich zu betrachten ist.

Die Bestimmung der Katalaseaktivität der sensiblen Zellen ließ feststellen, daß das Enzym im Verhältnis zur Vermehrung verspätet induziert wird [15] und bis zum Zustandekommen des anaeroben Milieus allmählich zunimmt (Abb. 1, Kurve 1/c). In der absolut Fucidin-resistenten Kultur wurde eine Katalaseaktivität überhaupt nicht beobachtet.

Aus dem Vergleich der die Atmungsintensität ausdrückenden Potentialkurven geht hervor, daß die O_2-Konsumption der resistenten und sensiblen Kulturen gleich ist. Ein wesentlicher Unterschied ergibt sich dagegen hinsichtlich der Katalaseaktivität. Beim Resistentwerden des sensitiven Stammes war bereits zu Beginn der Adaptation in Nährboden mit geringen Fucidin-Mengen (0,1 μg/ml) eine beträchtliche Verminderung der Katalaseaktivität zu beobachten. Bei zunehmender Fucidin-Konzentration läßt die Enzymaktivität weiter nach, um schließlich ganz aufzuhören (Abb. 2).

Besprechung

Unsere Befunde lassen sich damit erklären, daß das Eingreifen des Fucidins in den aeroben Mechanismus des Mikroorganismus bei den Katalase induzierenden Prozessen stattfindet. Eine entsprechende Fucidin-Konzentration hat die vollständige Aufhebung der Atmung und damit auch der Vermehrung der Mikroorganismen zur Folge.

In der fucidinfest gewordenen Kultur bedeutet die Aufhebung des Mechanismus des Katalasesystems nicht die Ausschaltung sämtlicher energieliefernder Prozesse. Die Wirkung des Fucidins manifestiert sich im Laufe der Adaptation in einer Verschiebung des Gleichgewichtes der nebeneinander funktionierenden Oxydationsvorgänge, d. h. daß zur Sicherung der erforderlichen Energie anstatt der mit Katalase funktionierenden energieproduzierenden Prozesse ein anderer Oxydationsmechanismus das Übergewicht erlangt.

Dieser besondere Effekt des Fucidins wird von anderen Antibiotika (z. B. Streptomycin, Erythromycin usw.) nicht ausgelöst und dementsprechend äußert sich deren antibakterielle Wirkung in der Hemmung eines anderen aeroben Mechanismus. Somit führt die Kombinierung dieser Antibiotika mit Fucidin unbedingt zur Erzielung einer intensiveren antibakteriellen Wirkung [16, 17].

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BACTERIOSTATIC EFFECT OF SALIVA OF CHILDREN WITH AND WITHOUT CARIES*

By

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Summary. The bacterial flora and bacteriostatic effect of caries resistant (CR) and caries active (CA) saliva has been examined. The normal flora of the mouth consists mainly of aerobic streptococci in CA and of anaerobic streptococci in CR patients. Saliva exerts a growth-inhibiting action chiefly on lactobacilli. CR saliva is more definitely bacteriostatic than CA. Both kinds of samples were only slightly inhibitory to streptococci.

HATTYASY *et al.* [10] and TÓTH [13] have shown that the oxygen uptake of saliva is higher in caries active (CA) than in caries resistant (CR) patients. Oxygen uptake by CR saliva sometimes started after a latency period, in other cases transient stagnations and even a decrease occurred. It has been assumed that these variations were due to differences in the bacterium content and bacteriostatic effect of various specimens. The opinions generally agree that, besides lysozyme, saliva contains another bacteriostatic principle [1—3, 7, 8, 11, 12, 14, 15]. The two substances can be differentiated on the basis of physical and chemical properties (solubility, filtrability, thermostability, etc.). Saliva itself is probably bacteriostatic, as samples obtained from the sterile submaxillary gland exert antibacterial activity [9, 16]. According to CLOUGH [6], there is no difference in lactobacterial inhibition between the saliva of CA and CR individuals. It seemed therefore, desirable to investigate the bacterium content and bacteriostatic activity the saliva of CA and CR patients.

Materials and methods

Selected caries active (CA) and caries resistant (CR), 6 to 14 year old boys were examined. Each group contained 4 boys, aged 6, 8, 9 and 13 years, respectively, in group CA and 6, 10, 11 and 14 years, respectively, in group CR.

Patients with considerably softened, not or slightly discoloured caries affecting at the time of examination at least 5 teeth, were regarded as belonging to group CA.

When inspection and X-ray examination revealed no caries or fillings, the patient was grouped as CR.

Saliva was taken before breakfast (a) with glass suction tubes from the sublingual region, (b) without stimulation, from sputum. On the day of the experiment the children were made to refrain from brushing their teeth.

Identification of bacteria. For bacteriological examination 0.1—0.2 ml sublingual saliva was taken with a pipette; interdental and carious debris was sampled with a loop.

* Presented in part at the 10th Congress of O. R. C. A. in Geneva July, 1964.

Bacterial counts were determined in semisolid agar and thioglycolate medium. Although the cultures formed fairly typical colonies in the above media, identification was performed on the usual blood agar and other media. Changes in the reaction of the media were measured by an electric pH meter.

The bacteriostatic effect was examined in the Jouan biophotometer. A clear casein hydrolysate medium providing good growth conditions to lactobacilli and other organisms was used. Anaerobic cultivation was performed in the same medium overlayered with liquid paraffin. Into the cuvette 6 ml medium, 3 ml saliva concentrated 1 : 3 *in vacuo* and 0.02 ml inoculum (20 million cells per ml) were pipetted. The concentrated saliva was irradiated previously with an U. V. lamp in order to decrease its living cell content below a level not interfering with the experiment.

Results

Oral flora. Streptococci predominated in the saliva of both groups of patients and in interdental and carious debris. Similar findings have been reported in the literature. The streptococcal strains fermented carbohydrates actively, grew well under aerobic and anaerobic conditions and produced a final pH of 5.0—5.3 in carbohydrate-free medium originally adjusted to pH 7.4. Acid production in carbohydrate-free medium was probably due to amino

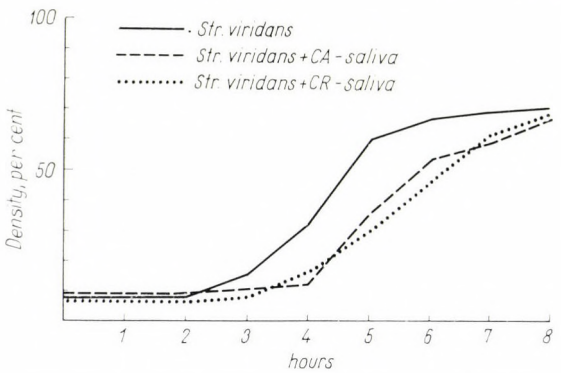

Fig. 1. Multiplication of oral *Str. viridans* was slightly inhibited by CA or CR saliva. There was no significant difference in the inhibitory activity of the two kinds of samples. The graph was prepared on the basis of biophotometer recording

acids formed in the course of protein decomposition. A similar degree of acid reaction in carbohydrate-free medium was produced only by lactobacilli.

There was a difference between the two groups in bacteria isolated from interdental and carious debris. In group CR mainly cultures growing only anaerobically occurred; strains isolated from CA patients generally multiplied under both aerobic and anaerobic conditions.

While in CA and CR saliva and in CR interdental debris lactobacilli were found in 10—14 per cent, the corresponding frequency of these organisms in carious teeth was only 2 per cent. Lactobacilli were isolated from 70 per cent of the examined patients. Of these cultures 30 per cent corresponded to *L. casei*, 60 per cent to *L. acidophilus* and 10 per cent to other lactobacilli.

Bacteriostatic activity of saliva. Some of the isolated and identified cultures were used for studying the bacteriostatic effect of CA and CR samples. Bacteriostasis was exerted only against *Str. viridans* and lactobacilli.

Photometrically measurable multiplication of *Str. viridans* commenced after 2 to 3 hours' incubation (Fig. 1). The increase in optical density in the control cuvette was linear for 5 to 6 hours. Multiplication in cuvettes containing CA or CR saliva was somewhat delayed, but after 8 hours the density of

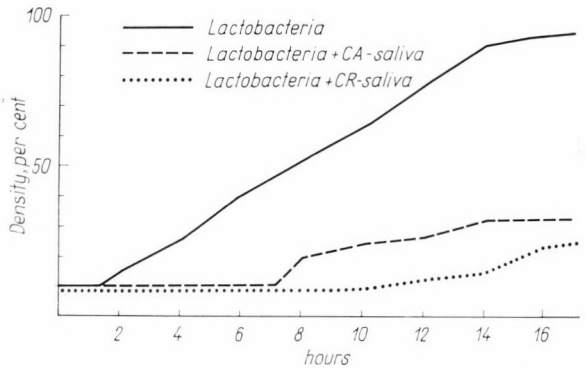

Fig. 2. Multiplication of oral lactobacilli was inhibited by both CA and CR saliva; the latter was more inhibitory. The graph was prepared on the basis of biophotometer recording

the suspension was similar to that measured in the control. No significant difference in bacteriostatic effect occurred between CA and CR samples. *L. acidophilus* and *casei* showed a linear increase in the control medium (Fig. 2). In cuvettes to which saliva had been added multiplication started only after 8 to 9 hours; after 18 hours density was still low. There was a considerable difference in this respect between CA and CR samples. In the presence of CA saliva growth commenced after 7 hours. CR saliva retarded the growth for 11 to 12 hours and the density remained lower throughout an 18-hour incubation period than that measured in the suspension containing CA saliva.

Discussion

It has been shown previously that the oxygen uptake of CA saliva is considerably higher than that of CR saliva. Bacteriological examinations have revealed that while in the saliva and interdental debris of CR individuals aerobic bacteria predominate, in the saliva and carious lesions of members of the CA group mainly aerobically cultivable streptococci are present. This finding may explain the difference in oxygen uptake.

It has been shown that acid-producing bacteria occurred at an approximately equal ratio in CA and in CR specimen. The incidence of *L. acidophilus* in carious teeth was insignificant.

Saliva inhibited the growth of lactobacteria. In contrast to CLOUGH's findings [6], we observed a difference in antibacterial activity between CA and CR saliva; CR was more definitely bacteriostatic.

From the results it may be concluded that for the lower "L. acidophilus index" in CR individuals the higher bacteriostatic activity of CR saliva is responsible at least partly [4, 5].

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STUDIES ON THE ANTIGENIC STRUCTURE OF MYCOBACTERIA

I. COMPARISON OF THE ANTIGENIC STRUCTURE OF PATHOGENIC AND SAPROPHYTIC MYCOBACTERIA

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Summary. The antigenic structure of 1 human, 4 bovine and 4 avian type *Mycobacterium tuberculosis*, 2 *M. paratuberculosis*, 1 *M. phlei*, 1 *M. smegmatis* and 1 *M. minetti* strains has been examined. Immune sera were prepared in rabbits with ultrasonic disintegrated bacteria emulsified in Freund's adjuvant. The number of antigenic components, their electrophoretic mobility and the occurrence of components in other types or species were determined by *Ouchterlony*'s and *Graber* and *Williams*' methods. In *M. tuberculosis* human type 10, in bovine type 8—10, in avian type 8—9 antigen components were distinguished. Saprophytic mycobacteria yielded 4—6 components. Comparative examinations with human and avian *M. tuberculosis* immune sera indicated that at least one common antigen was shared by all examined mycobacteria. Specific factors were also revealed for each species. Pathogenic strains contained a common component which was absent from saprophytic mycobacteria.

Classification into species and types of organisms belonging to the Mycobacterium genus is important for differentiation purposes among saprophytic cultures and strains adapted to various hosts, and in view of the biological, biochemical and antigenic differences existing among the taxonomic units.

Detailed analysis of biochemical properties greatly improved the identification of attenuated and avirulent strains and the recognition of biological mutations occurring in consequence of drug treatment of human patients.

Differentiation of mycobacteria on the basis of antigenic structure has been attempted for several decades. Examinations carried out with various methods were generally focussed on a few antigenic components, therefore incomplete and often incomparable data are only available.

The complement fixation test was used for the examination of antigens extracted in different manners [4, 7, 8, 14, 15, 23]. Although common antigens were revealed in various species of mycobacteria, the methods failed to give information as to the whole antigenic structure and were therefore of a limited value in differentiation.

MIDDLEBROOK and DUBOS' haemagglutination test and its several modifications have made it possible to identify certain antigenic components in pathogenic and saprophytic mycobacteria. Thus, common antigens were found in human type *M. tuberculosis*, BCG and *M. phlei* [21]. By absorption combined with haemagglutination in atypical photochromogenic mycobacteria a, b and c, in human type *M. tuberculosis* b and c, in *M. smegmatis* c antigenic factors were distinguished [2]. Using the same method A, B, C and D factors

were shown in human type *M. tuberculosis*, A, B, and C factors in *M. phlei* and A, B and D factors in *M. johnei* [9].

A great progress has been made by the use of the agar gel precipitation and immunochemical methods. With OUCHTERLONY's agar gel precipitation technique PARLETT and YOUMANS [17] examined a number of mycobacterial strains. They used the concentrated filtrate of cultures as antigen and divided the examined strains into 4 groups. In group I including virulent and avirulent *M. tuberculosis*, 20 atypical human strains and 1 BCG, 1 *M. avium* and 1 *M. minetti* cultures, four different antigenic components were revealed. In group II containing 1 *M. avium* and 1 bovin mycobacterium, two antigens were determined. These antigens were identical with two of the antigens present in group I strains. In group III, four different antigens were distinguished. Saprophytic strains belonging to this group gave no cross reaction with group I and II antigens.

In subsequent examinations PARLETT and YOUMANS used living bacteria mixed into agar. With this technique they were able to divide the examined 98 mycobacterial strains into 8 antigenic groups.

In other experiments carried out with the agar gel method, extracted antigens were applied [5, 11]. It has been demonstrated that pathogenic mycobacteria contain special components that distinguish them from saprophytic strains. The examined cultures shared at least one common component.

By immune electrophoresis, PENSO [19] examined human, bovine and saprophytic mycobacteria. He demonstrated that human and bovine *M. tuberculosis* comprised one homogeneous group. With the exeption of one, all components were found in both types. Every bovine strain contained a special factor absent from human strains. In each mycobacterial species characteristic antigenic components were found. One component was present in all the examined strains.

According to the results of various authors, the mycobacterial species are antigenically more or less related. The relationships are not yet clear in all details, especially those of the common and species specific antigen components.

Complete elucidation of the antigenic structure of mycobacteria responsible for human and animal infections is of great importance in practical epidemiology, since for the detection of the aetiological agents by serum antibody determination the knowledge of the complete antigenic structure is needed.

Materials and methods

Antigens. Four week Sauton cultures of mycobacteria were treated with 3 per cent phenol for 16 hours. After filtration the bacteria were suspended in saline at 300 mg/ml amounts and subjected in ice bath to ultrasonic treatment for 15 minutes. To the opalescent supernatant obtained after centrifugation at 3 000 r.p.m., merthiolate (1 : 10 000) was added. The antigens

were stored at 4°C. The following strains were used: *M. tuberculosis* human type H_{37}, bovine type Rv, Gräub, BCG, VR. I, avian type G. H., A. I., Gotar I, G. B. I., *M. paratuberculosis* W. 395, J., *M. phlei* S. 1, *M. minetti* S. 070, *M. smegmatis* S. 10.

Immune sera. The nitrogen content of antigens prepared as described above was determined with KJELDAHL's method. Each antigen was so diluted as to contain 5 per cent total protein. Then the antigen was emulsified with incomplete FREUND's adjuvant (50 per cent antigen solution, 10 per cent Arlacel A and 40 per cent Bayol F paraffin). Rabbits were six times injected subcutaneously with 1 ml of the emulsion at weekly intervals. The animals were bled 14—24 days after the last injection. The following strains were used for immune serum production: *M. tuberculosis* bovine type Gräub, BCG, VR. I, avian type G. H., A. I., Gotar I, G. B. I., *M. paratuberculosis* W. 395, J., *M. phlei* S. 1., *M. minetti* S. 070, *M. smegmatis* S. 10.

Agar gel diffusion experiments were performed as described by OUCHTERLONY [16]. Plates 4 mm tick were poured into defatted petri dishes with 2 per cent agar containing 0.9 per cent NaCl and 0.01 per cent merthiolate. Serum reservoirs were prepared at 7 mm distances. After pipetting the examined materials into the reservoirs, the petri dishes were placed in a wet chamber and incubated at 37° C for 24 hours.

Immune electrophoresis. As described by GRABAR and WILLIAMS [6], a 2 per cent agar solution of 70°C, containing pH 8.6 veronal buffer at ionic strength 0.05 was poured onto 90 × 60 mm glass plates so as to give layers 4 mm thick. After cutting out the antigen reservoir, the antigen was warmed up to 40°C in a water bath, mixed with an equal amount of 2 per cent buffer-free agar cooled previously to 40°C, and pipetted into the reservoir. Electrophoresis was performed at 3—4 V and 0.8 mA/cm of agar for 3 hours. Then a 3 mm wide diffusion reservoir was prepared and filled with immune serum. The plates were placed in a wet chamber and incubated at room temperature for 48 hours. Electrophoretic mobility of the antigen components was caculated by dividing the distance between the starting place and centre of the precipitation line with the time and potencial drop.

At first the number of antigenic components and their electrophoretic mobility in homologous systems were determined, then the presence of common antigens in various species and types was examined in heterologous systems.

Results

Immune serum for human type *M. tuberculosis* strain H37 gave 10 precipitation lines with the homologous antigen, 10 lines with bovine strains Rv and VR. I., and 8 lines with strains BCG and Gräub (Fig. 1). Five lines were obtained with avian strain GH 4 and with *M. paratuberculosis* strain W.

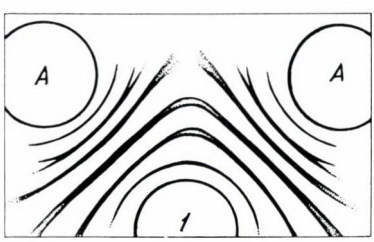

Fig. 1. A = Serum *M. tuberculosis* bovine strain Rv; 1 = Antigen *M. tuberculosis* bovine strain Rv

In both bovine strains Rv and V. R. I the homologous immune sera revealed 10 antigenic components. In strains BCG and Gräub, 8 components were found (Figs 2 and 3). Serum Rv gave 3 or 4 lines with avian, 5 lines with *M. paratuberculosis*, 2 lines with *M. phlei*, 2 lines with *M. smegmatis*, and 4 lines with *M. minetti* antigens (Figs 4 and 5).

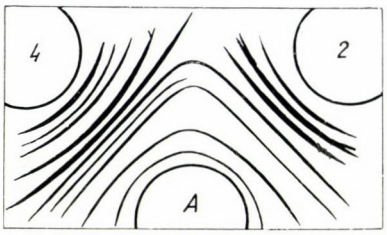

Fig. 2. A = Serum *M. tuberculosis* bovine strain Rv; 4 = Antigen *M. tuberculosis* bovine strain V. R. I.; 2 = Antigen *M. tuberculosis* bovine strain Gräub

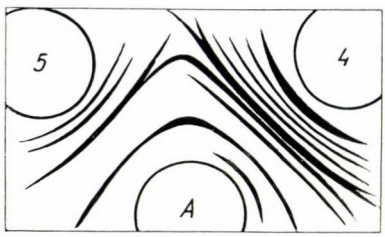

Fig. 3. A = Serum *M. tuberculosis* bovine strain Rv; 4 = Antigen *M. tuberculosis* bovine strain V. R. I.; 5 = Antigen *M. tuberculosis* avian strain GH

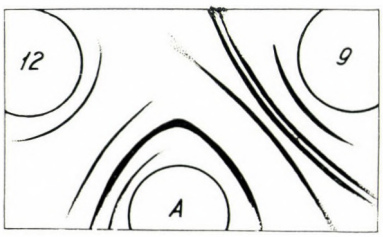

Fig. 4. A = Serum *M. tuberculosis* bovine strain Rv; 12 = Antigen *M. minetti*; 9 = Antigen *M. paratuberculosis*

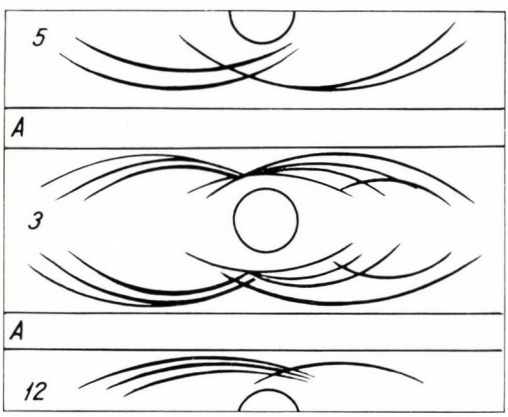

Fig. 5. A = Serum *M. tuberculosis* bovine strain Rv; 5 = Antigen *M. tuberculosis* avian strain GH; 3 = Antigen BCG; 12 = Antigen *M. minetti*

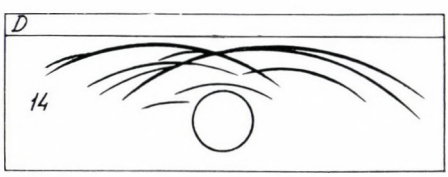

Fig. 6. D = Serum *M. tuberculosis* bovine strain Rv; 14 = Antigen *M. tuberculosis* human strain H_{37}

Immune reactions obtained with electrophoretic analysis are presented in Fig. 6.

On the basis of eletrophoretic mobility, the antigenic fractions were designated in alphabetical order, namely those travelling towards the anode with capitals, those travelling towards the cathode with small letters. It should be noted that components designated with the same letter are antigenically identical only in reactions obtained with one type serum; in other words, the same letter represents different antigens in Table I (human serum) and in Table II (avian serum).

M. tuberculosis human and bovine type antigens present in the homologous organisms and in other types and species are shown in Table I.

Table I

Antigenic components of mycobacteria obtained with serum M. tuberculosis human type

	g	f	e	d	c	b	a	A	B	C
Human H37	+	+	+	+	+	+	+	+	+	+
Bovine Rv	+	+	+	+	+	+	+	+	+	+
Bovine Gräub		+	+	+		+	+	+	+	+
Bovine V. R. I. ...	+	+	+	+	+	+	+	+	+	+
BCG	+	+	+			+	+	+	+	+
Avian GH			+	+		+			+	
Avian AI			+	+		+				
Paratuberculosis W.			+		+	+		+		+
Paratuberculosis J.			+		+	+		+		+
M. phlei SI.						+			+	
M. smegmatis S10						+			+	
M. minetti S070					+	+	+		+	

With *M. tuberculosis* avian strains the homologous immune serum revealed 9 antigenic fractions, except for strain AI, which contained only 8 components (Fig. 7).

As to heterologous systems, the immune sera prepared against *M. tuberculosis* avian strain GH gave 4 lines with human H37, 6 lines with paratuberculosis, 2 lines with phlei, 2 lines with smegmatis and 3 lines with minetti antigens (Fig. 8).

Immune reactions of electrophoretically separated antigenic components are presented in Fig. 9.

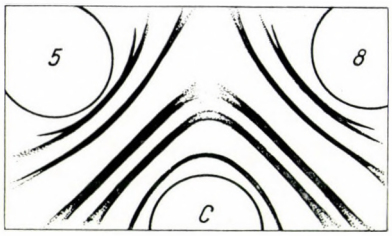

Fig. 7. C = Serum *M. tuberculosis* avian strain GH; 5 = Antigen *M. tuberculosis* avian strain GH; 8 = Antigen *M. tuberculosis* avian strain GB. I.

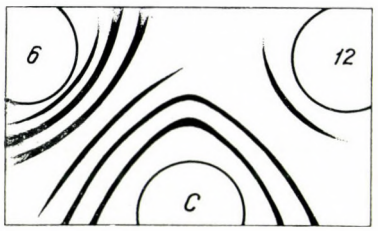

Fig. 8. C = Serum *M. tuberculosis* avian strain GH; 6 = Antigen *M. tuberculosis* avian strain A. I.; 12 = Antigen *M. minetti*

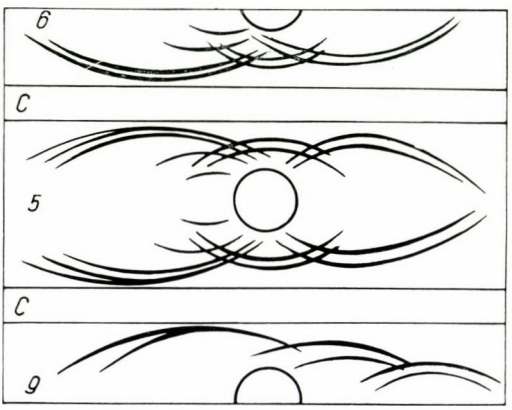

Fig. 9. C = Serum *M. tuberculosis* avian strain GH; 6 = Antigen *M. tuberculosis* avian strain A. I.; 5 = Antigen *M. tuberculosis* avian strain GH; 9 = Antigen *M. paratuberculosis* strain W

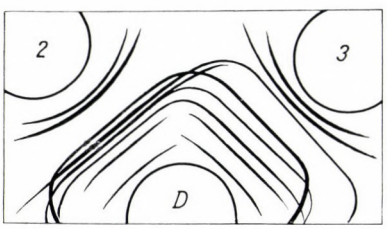

Fig. 10. D = Serum *M. tuberculosis* human strain, H_{37}; 2 = Antigen *M. tuberculosis* bovine strain Gräub; 3 = Antigen BCG

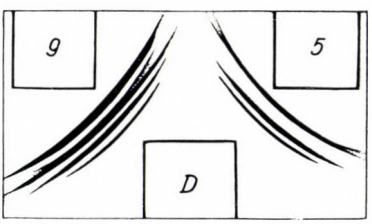

Fig. 11. D = Serum *M. tuberculosis* human strain, H_{37}; 9 = Antigen *M. paratuberculosis* strain, W; 5 = Antigen *M. tuberculosis* avian strain GH

M. tuberculosis avian type antigens present in the homologous organisms and other types and species are demonstrated in Table II.

In *M. paratuberculosis* 10 components were shown. Six of these factors were present also in *M. tuberculosis* avian type strains.

Table II

Antigenic components of mycobacteria obtained with serum M. tuberculosis avian type

	f	e	d	c	b	a	A	B	C
Avian GH.	+	+	+	+	+	+	+	+	+
Avian AI.	+		+	+	+	+	+	+	+
Avian Gotar I.	+	+	+	+	+	+	+	+	+
Avian G. B. I.	+	+	+	+	+	+	+	+	+
Human H 37		+	+		+			+	
Bovine Rv		+	+		+			+	
Paratuberculosis W.		+	+		+	+	+		+
Paratuberculosis J.		+	+		+	+	+		+
M. phlei SI.			+		+				
M. smegmatis S 10.			+		+				
C. minetti S 070.				+	+		+		

Of paratuberculosis antigens 2, 2 and 3 were revealed in *M. phlei*, *M. smegmatis* and *M. minetti*, respectively.

With the homologous immune sera in *M. phlei* 5, in *M. smegmatis* 6, antigenic components were distinguished.

Discussion

When examining and comparing the antigenic structure of mycobacteria, special attention has been paid to the use of extraction methods causing no important biochemical alteration in the antigens. Heating or chemicals may be highly injurious especially to protein components and may cause the loss of species of type specific factors. Therefore, the antigens were extracted by ultrasonic disintegration.

It has been shown that in mycobacteria belonging to various species and types, different numbers of antigenic components are present. Human and bovine type *M. tuberculosis* strains, however, were shown to contain identical antigens.

The number of antigenic components is characteristic of one strain only, as some, although slight, intraspecies and intratype differences have been demonstrated. Thus, of the bovine type strains BCG and Gräub, and of the avian strains A. I. contained only 8 factors.

As shown in Tables I and II, some components were found only in the homologous species or type; others were present also in other species or types. One component was shared by all examined mycobacteria.

If a direct association is assumed to exist between the number of common antigenic factors and the degree of relationship, bovine *M. tuberculosis* is more closely related to *M. paratuberculosis* than to avian *M. tuberculosis*.

A close antigenic relationship was found to exist between avian *M. tuberculosis* and *M. paratuberculosis*, as 6 of the avian components were present in paratubercle bacteria. This finding corresponds to diagnostic observations indicating that in paratuberculotic infections avian tuberculin produces a stronger allergic reaction than mammalian tuberculin.

In saprophytic species only 2 or 3 bovine antigen components were revealed.

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ELECTRONMICROSCOPIC EXAMINATION OF KB CELL CULTURES INFECTED WITH ADENOVIRUS TYPE 12*

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Summary. Infection of KB cells with adenovirus type 12 resulted in the appearance in the nucleus of a substance characterized by the absence of nucleic acids, resistance to pepsine digestion, osmophilia and high electron density. The amount of this substance increased rapidly; in 36 to 48 hours it formed a coherent reticular pattern covering the total nucleus. At certain points hexagonal, crystal-like structures appeared.

The neighbourhood of the above reticulum was successively filled by virus particles until virus crystals of variable sizes had formed. In some preparations the total nucleus was stuffed with viruses. Parallel to the progress of cell damage one or more viruses, sometimes even complete virus crystals appeared in the cytoplasm.

The average diameter of an individual virus was 70 mμ as measured in ultra-thin sections of cells embedded in Epon. The round or ovoid viruses comprised a central, osmophilic and electron dense part (nucleoid) surrounded by a less dense viroplasm with distinct borders. The nucleoid was sensitive to desoxyribonuclease and resistant to ribonuclease. Pepsin treatment resulted in digestion of all visible constituents but the nucleoid.

In the cytoplasm of infected cells we could occasionally observe the formation of a highly organized, rich membrane pattern.

Aspecific degenerative changes elicited by the infection were also observed.

The cytopathological changes induced by adenovirus type 12 resembled mostly those elicited by type 5 of the same agent.

Since the isolation of the first adenovirus by ROWE *et al.* in 1953 [1], a remarkable amount of information has been accumulated about this group of agents. Their ability to persist in a latent form for prolonged periods in different parts of the organism, particularly in the lymphatic tissues [2, 3] appeared to be of interest. A further important phenomenon was the high intrapulmonal oncogenicity of type 12 adenovirus in newborn hamsters (41 out of 45 infected animals developed tumour) reported by TRENTIN *et al.* [4]. This observation has been supported by similar findings in newborn mice and rats treated with the same agent. Other types of adenovirus were also found to possess oncogenic properties [5, 6].

These observations have given rise to the morphological study of the pathological events elicited at the cellular level by the adenoviruses. The general importance of these studies is quite clear in view of the virus-tumour relationship.

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The present paper is a report on a series of examinations on KB cells infected with adenovirus type 12. The replication, morphology, chemical composition and cytopathology of the agent have been studied in semi-thick and ultra-thin sections.

Materials and methods

The KB cell strain were used throughout. Stationary cultures were prepared in bottles using a total of 10 ml fluid containing 5×10^5 cells/ml. The growth medium consisted of 0.5 per cent lactalbumin hydrolysate, 0.1 per cent yeast extract, and 10 per cent calf serum in Earle's nutrient fluid. After appropriate incubation the cultures were infected with 0.1 ml of a suspension of the prototype strain of adenovirus type 12 containing 10^6 $TCID_{50}$/ml. Simultaneously with the infection the medium was replaced with one with a 6 times higher hydrocarbonate content.

Cells of one bottle each were processed for electron microscopy after 12, 24, 36, 48, 72, 96, 120, 144 and 168 hours following infection. Control cultures of identical batch number and type were processed simultaneously. Each experiment was performed in triplicate.

The procedures of fixation and embedding are given in Table I.

Table I

Methods of fixation and embedding applied in the experiments

	Fixation				*Embedding*
1.	2 % osmium tetroxide	Phosphate-buffered (Millonig II)	at pH 7.3	1 hour	Epon
2.	6.25 % glutaraldehyde	"	"	20 minutes	Epon
	2 % osmium tetroxide (post fixation)	"	"	1 hour	
3.	6.25 % glutaraldehyde	"	"	20 minutes	GMA
4.	10 % formol	"	"	20 minutes	GMA
5.	5 % formol and 5 % acrolein	"	"	20 minutes	GMA

Preparations fixed in glutaraldehyde and embedded in glycol methacrylate (GMA) were used for different enzyme digestion assays as listed in Table II.

Table II

Solutions and methods of enzymatic digestions

1.	0.1 and 0.5 % pepsin in 0.1 *N* HCl	—	37°C	20 minutes — 1 hour
2.	0.1 % ribonuclease (RNase) in distilled water, adjusted to	pH 6.8	37°C	1 hour
3.	0.1 % deoxyribonuclease (DNase) in distilled water, adjusted to	pH 6.2—6.5	37°C	1 hour — 4 hours
4.	0.1 % *N* HCl	—	37°C	20 minutes — 1 hour
5.	distilled water	pH 6.2—6.8	37°C	1 hour — 4 hours

As staining procedures either the uranyl acetate or KARNOVSKY's plumbit method [41] or a combination of the two were applied. The electron microscope was a Siemens Elmiscop I apparatus (80 KW and 50 μ objective aperture).

In addition to the ultrathin ones sections 1 to 2 μ thick were also prepared. These were examined under the light microscope after staining with a mixture of Azur II and methylene blue or in some cases by the Feulgen method.

Experimental

Electronmicroscopic morphology of non-infected KB cells. Cultures of KB strain consist of large, sometimes elongated or pleomorphic cells (Fig. 1). The cells have a large nucleus surrounded by a thin cytoplasmic layer. The rich chromatin matter is evenly distributed throughout the nucleus. The nucleoli (one or two) are large round or oval in shape and often of peripheral localization. The cytoplasm is rich in free ribosomes, while endoplasmic reticulum membranes are relatively scarce. A few oval or elongated medium-sized mitochondria are present in the cytoplasm. The Golgi apparatus has the usual pattern and varies as to development. The cytoplasm occasionally contains some small osmophilic lipid droplets. The surface of the cells carries numerous thin microvilli of varying size.

Ultrastructure of infected cells. In infected cultures incubated for 12 hours the nuclei of several cells exhibited discrete focal condensations of chromatin granules and swollen nucleoli. In 24 hours there appeared in the nucleus irregular clumps of an intensively osmophilic, amorphous or finely granular substance (Fig. 2). Its amount increased rapidly and 36 hours after the infection the clumps were already forming a coherent network in 10 to 15 per cent of the cells.

Forty-eight hours after the infection small, round and dense virus particles appeared in varying numbers in the environment of the highly electron dense, osmophilic network which in some cases assumed a sharply marginated, hexagonal crystalline array (Figures 3a and 3b). Around the viral particles the nucleoplasm became less rich in chromatine and the formation of a light, less electron dense halo was observable. Simultaneously with these events a swelling of the nucleoli took place in the infected cells. Nuclei of the infected cells were often characterized by numerous cytoplasm-filled invaginations of their membranes. No particular ultrastructural lesions were demonstrable at that time in the cytoplasm.

The round or oval virus particles measured 70 mμ in diameter. Their number increased rapidly in the nuclei of infected cells. They were arranged either in irregular groups or in a crystalline array (Fig. 4), in the vicinity of the osmophilic, electron dense amorphous substance. The chromatin was pushed successively to the margin of the nucleus, near to the membrane. Quite often the nucleus was completely filled by viruses. The swollen nucleoli remained clearly recognizable (Fig. 5) even in these cases. Nevertheless, occasional granulation or segregation of the nucleolar components was also observed (Figs 6 and 7).

Parallel to the increase in the number of viruses and the formation of viral crystals in the nucleus, the cytoplasm exhibited definite signs of degeneration. The number of ribosomes decreased, the mitochondria became swollen

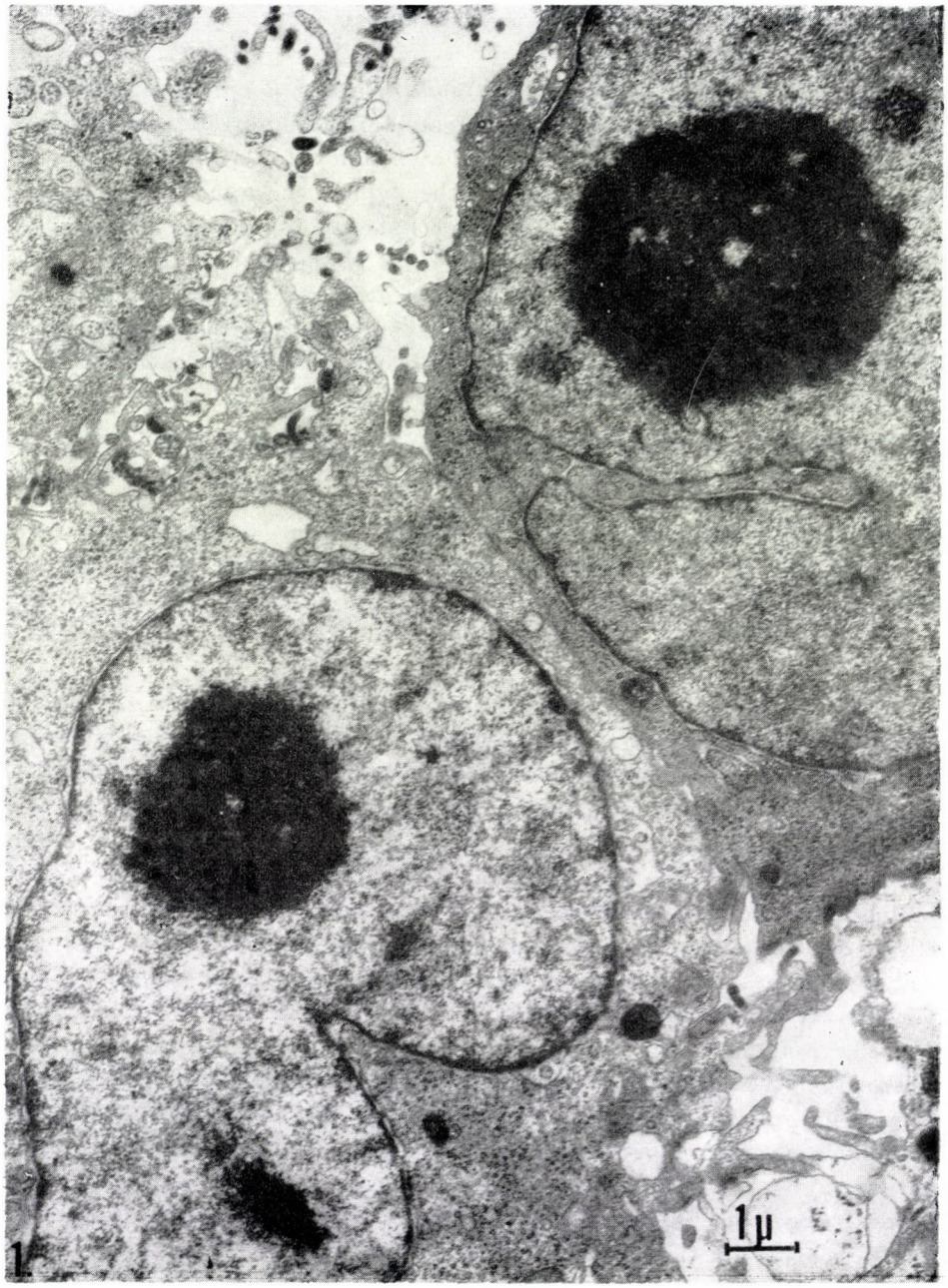

Fig. 1. Untreated KB cells from a six-day-old culture. Glutaraldehyde and osmium tetroxide fixation. Epon-embedding. Uranyl acetate and Karnovsky staining. Magnification, ×8000

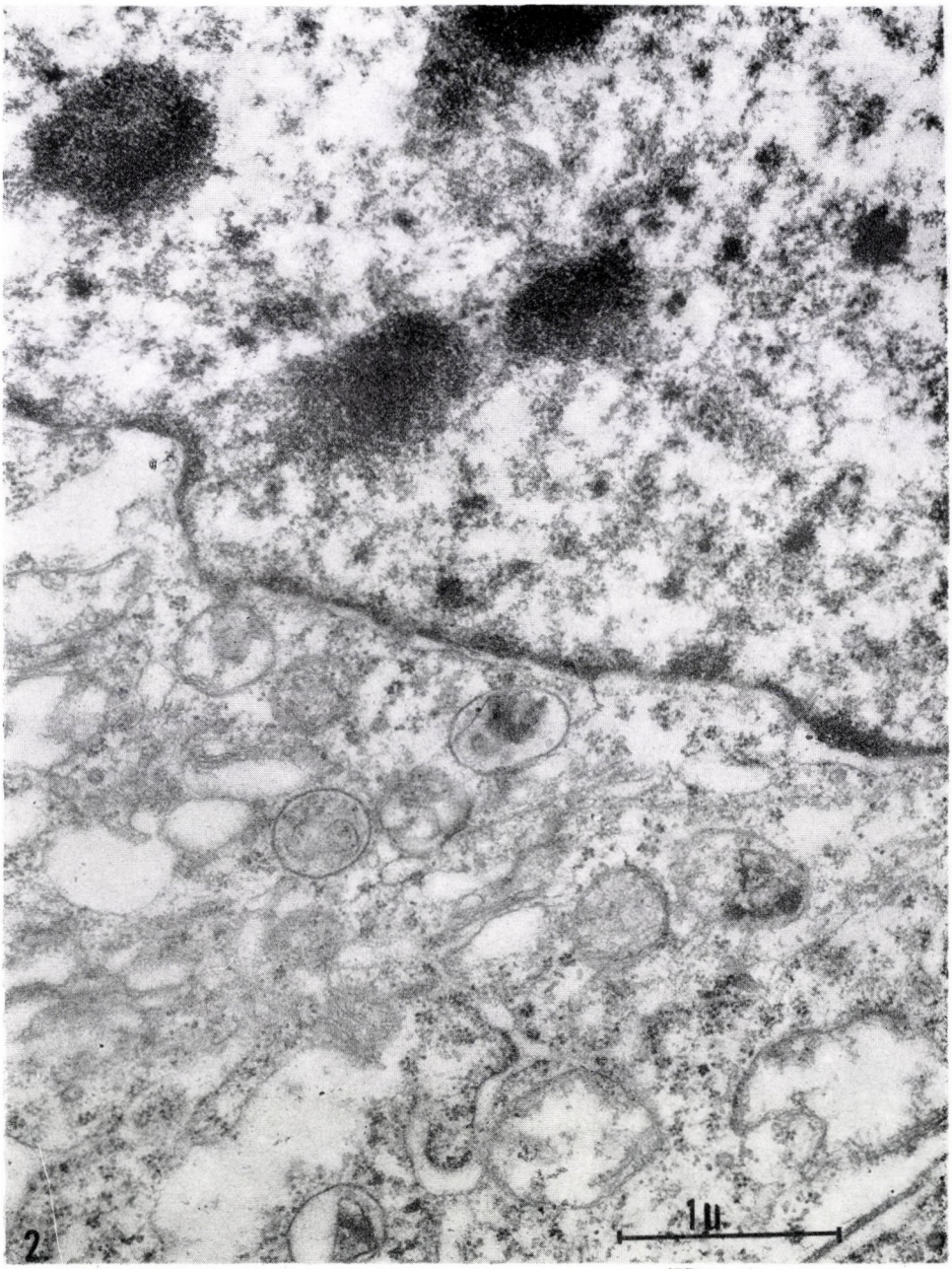

Fig. 2. KB cell 36 hours after infection with type 12 adenovirus. Irregular aggregates of an electron dense, osmophilic substance in the nucleus. Osmium tetroxide fixation. Epon embedding. Uranyl acetate and Karnovsky staining. Magnification × 30 000

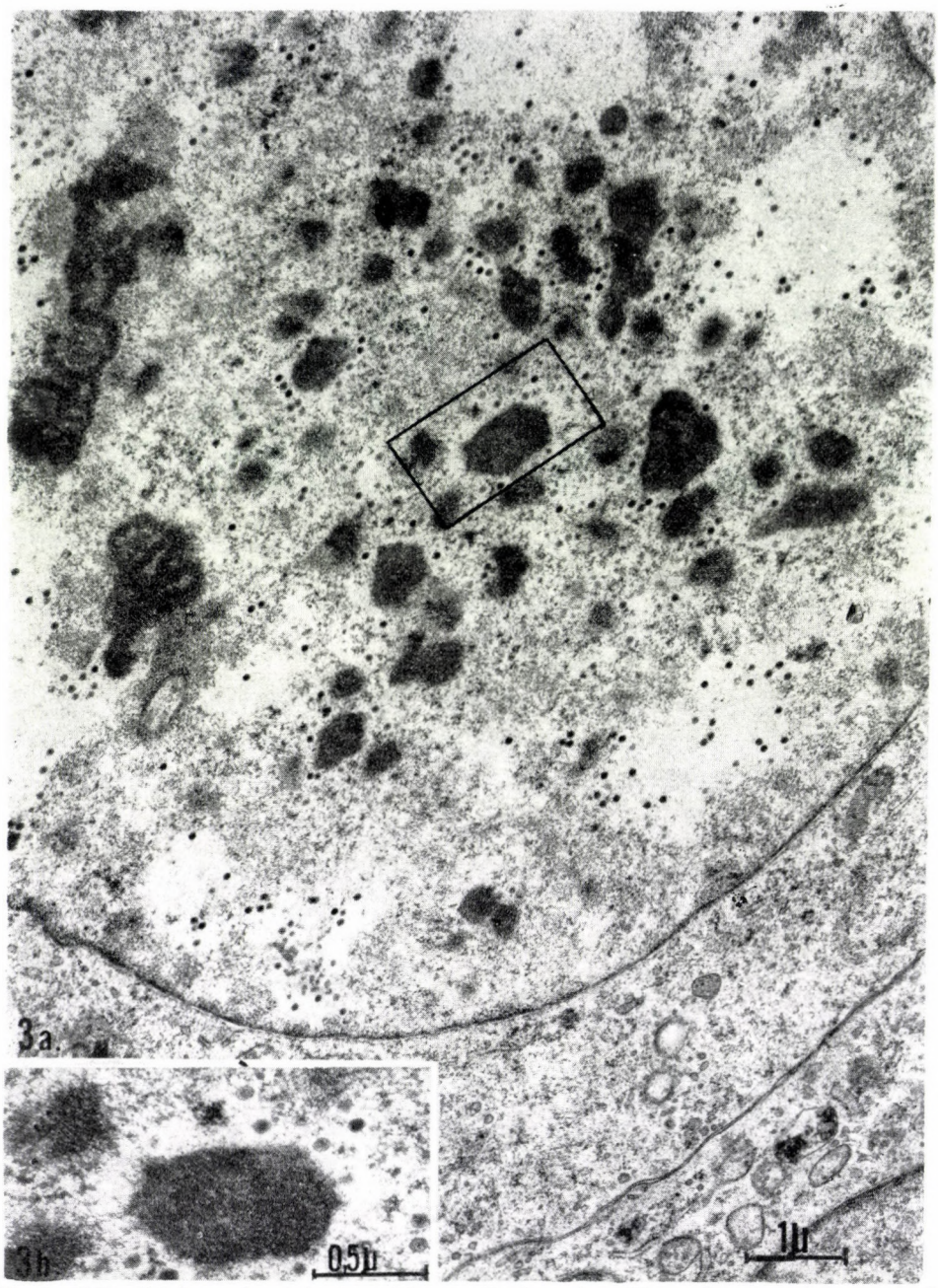

Fig. 3a. KB-cell 3 days after infection with type 12 adenovirus. The nucleus contains a network of highly electron dense, amorphous substance with occasional hexagonal crystalline arrays (framed). Viruses present themselves as dense spherical particles within the nucleus. Certain regions of the nucleus contain few chromatin. Osmium tetroxide fixation. Epon embedding. Uranyl acetate and Karnovsky staining. Magnification × 12 000

Fig. 3b. The hexagonal crystalline structure at higher magnification. Some single particles have a well-defined internal structure. Osmium tetroxide fixation. Epon embedding. Uranyl acetate and Karnovsky staining. Magnification × 30 000

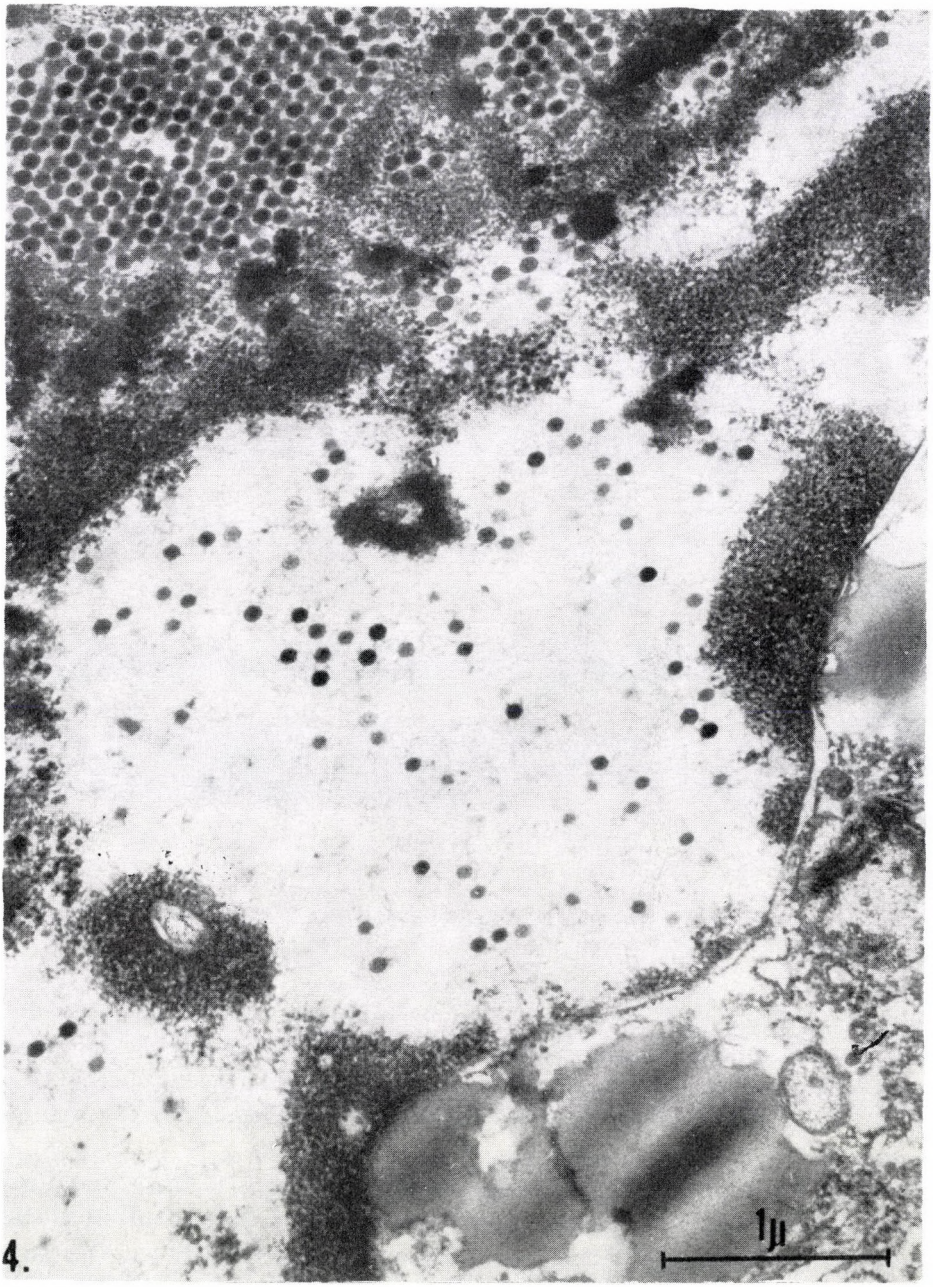

Fig. 4. KB cells eight days after infection with type 12 adenovirus. Crystalline array of viruses and osmophilic network in the nucleus. The chromatin has undergone marginization. Lipid droplets in the cytoplasm. Osmium tetroxide fixation, Epon embedding. Uranyl acetate and Karnovsky staining. Magnification $\times 30\,000$

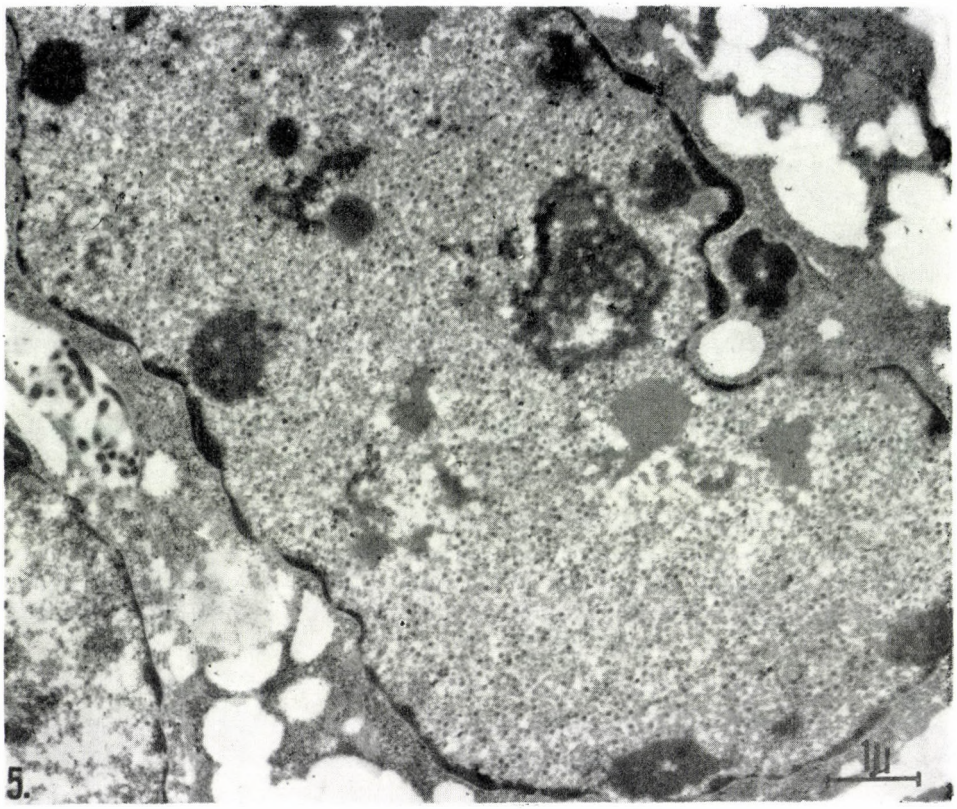

Fig. 5. KB cell 5 days after infection with type 12 adenovirus. The nucleus is transformed into a bag filled with virus. Remnants of the hypertrophic nucleolus and of the electron dense, amorphous matter are still recognizable. Marginization of chromatin. Formalin fixation. GMA-embedding. Uranyl acetate and Karnovsky staining. Magnification × 12 000

and lost their internal structures. Vacuoles of different size and osmophilic lipid granules appeared in the cytoplasm (Figs 4 and 8). Despite the apparently intact nuclear membrane some virus particles appeared in the cytoplasm. Occasionally large viral crystals could be observed in the cytoplasm, as a sign of a damage to the nuclear membrane.

In the cytoplasm of some infected cells there was a highly organized, extensive membrane system containing both rough and smooth surfaced lamellae (Fig. 10). These membranes form very regular parallel tubular structures which appear in longitudinal or vertical sections in the preparations.

The chemical characterization of the individual ultrastructural elements *in situ* has been made possible by the use of aldehyde fixatives and GMA embedding [7, 8]. These procedures have been applied in the present experiments for the characterization of the amorphous, highly electron dense material of the

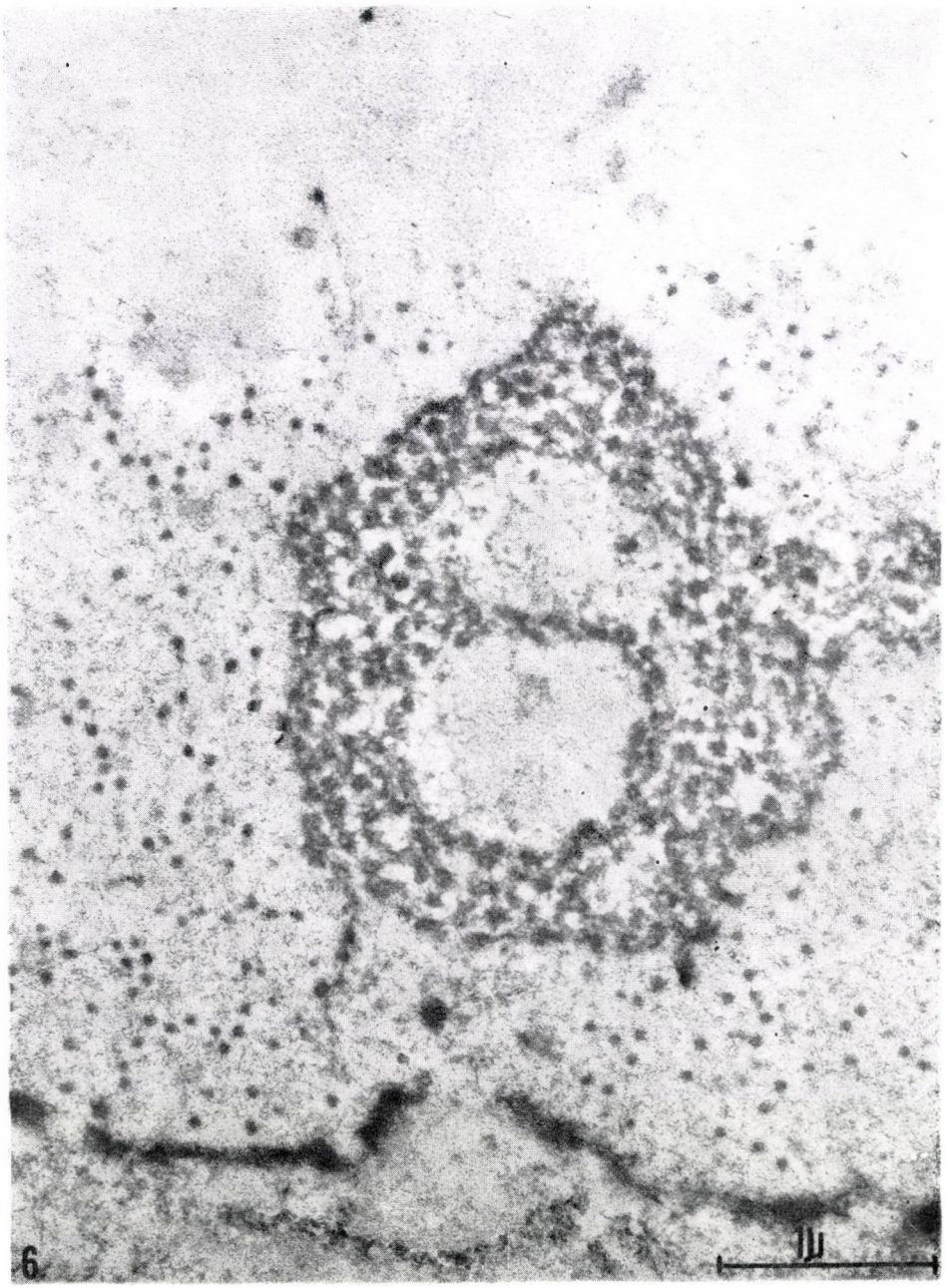

Fig. 6. KB cell 5 days after infection with type 12 adenovirus. In some cells the nucleolus is roughly granulated. This structural feature is more pronounced after pepsin treatment. The proteinaceous matrix of the nucleoplasm has been digested and from the viruses there remains only the nucleoid. The chromatin has undergone marginization. Formalin fixation. GMA-embedding. The preparations were digested with 0.5 per cent pepsin for 30 minutes. Magnification $\times 30\,000$

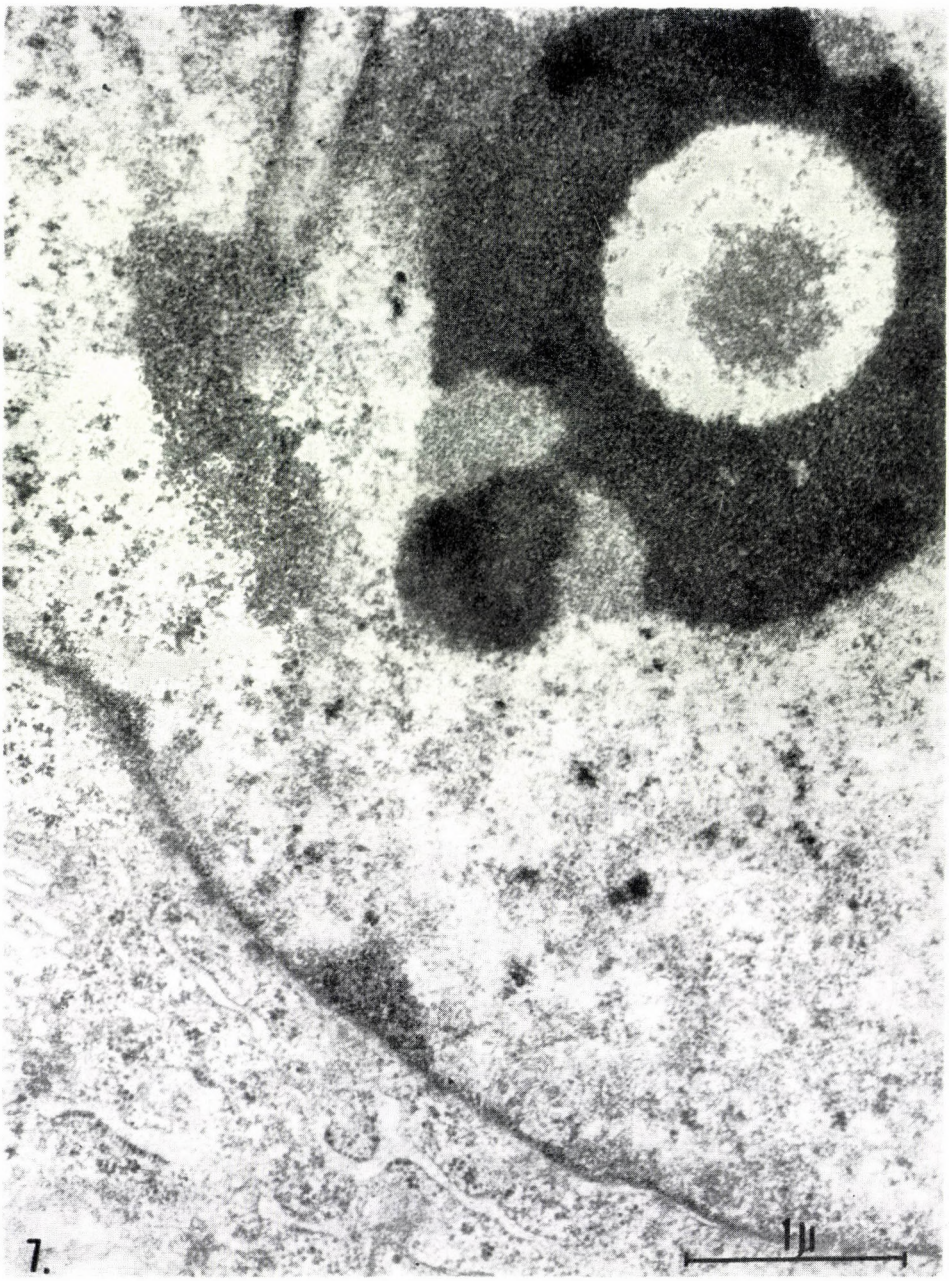

Fig. 7. KB cell five days after infection with type 12 adenovirus. A particular segregation of the components of the nucleolus is apparent. Osmium tetroxide fixation. Epon embedding. Uranyl acetate and Karnovsky staining. Magnification × 30 000

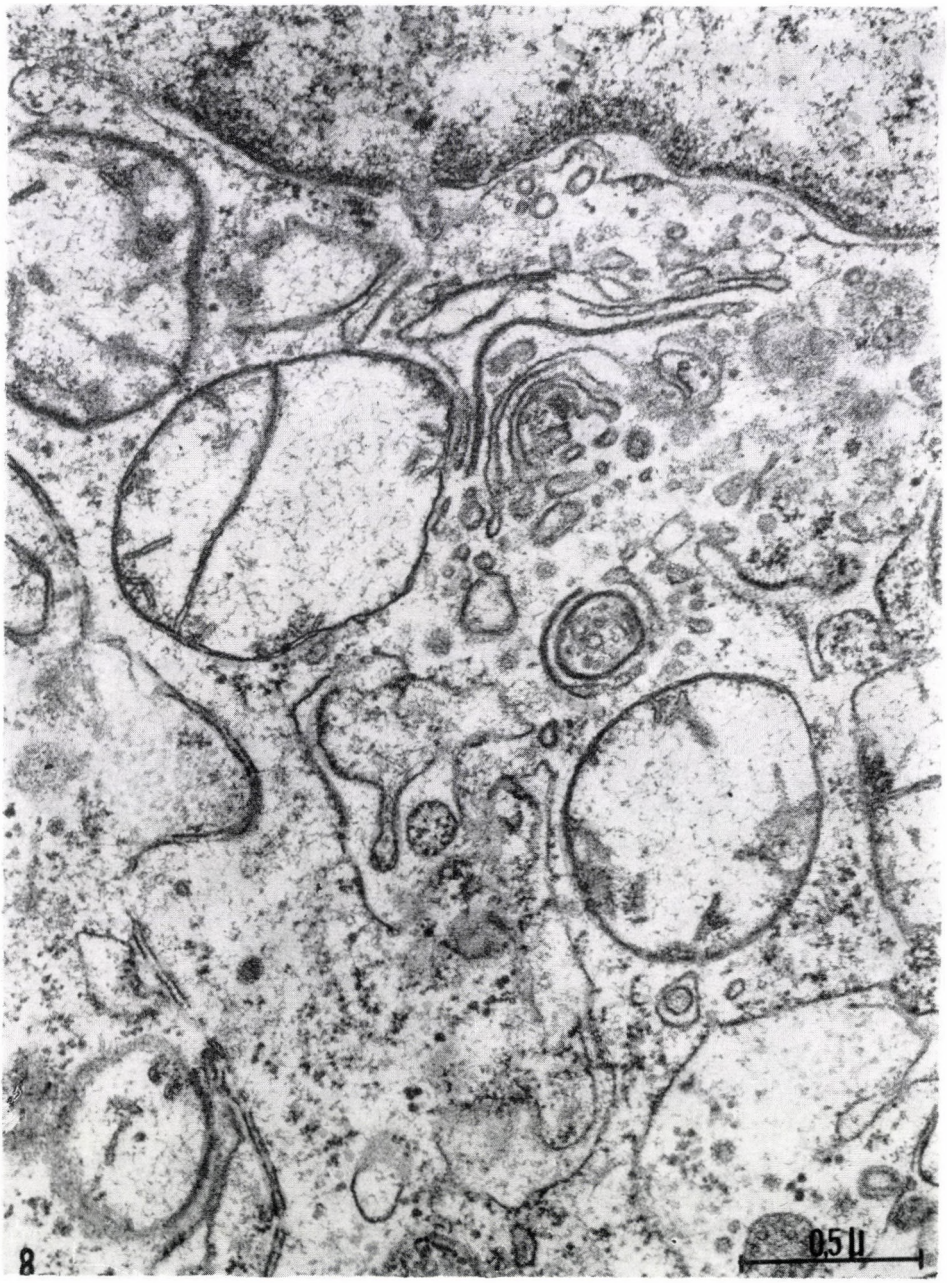

Fig. 8. KB cell five days after infection with type 12 adenovirus. Degenerative lesions of the cytoplasm (swollen mitochondria, disappearance of crystals). Osmium tetroxide fixation. Epon embedding. Uranyl acetate and Karnovsky staining. Magnification × 45 000

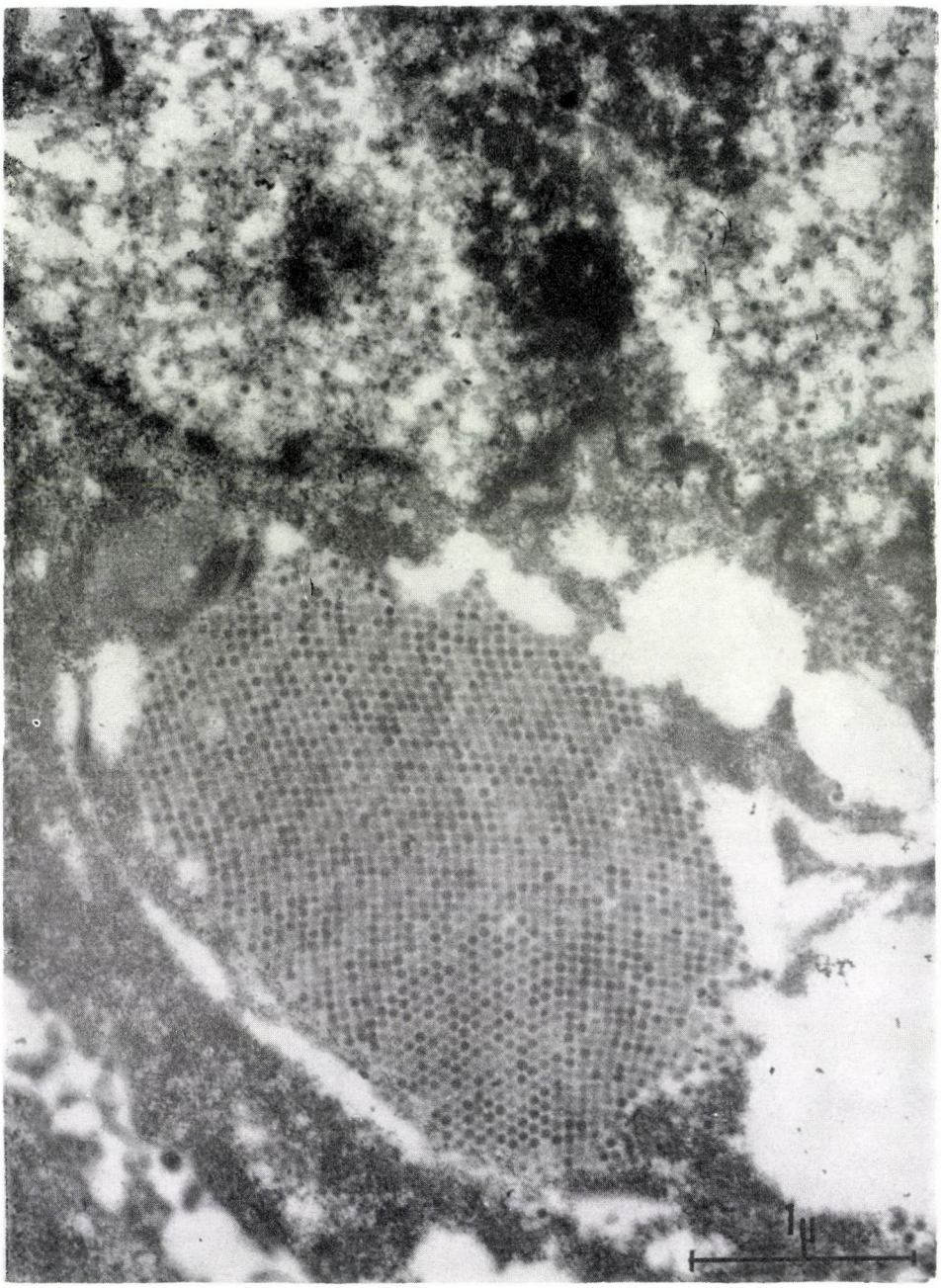

Fig. 9. KB-cell five days after infection with type 12 adenovirus. A debris of virus crystals is apparent in the cytoplasm of a cell with degenerative lesions. Some viruses are still seen in the nucleus. Formalin fixation. GMA embedding. Uranyl acetate and Karnovsky staining. Magnification $\times 30\,000$

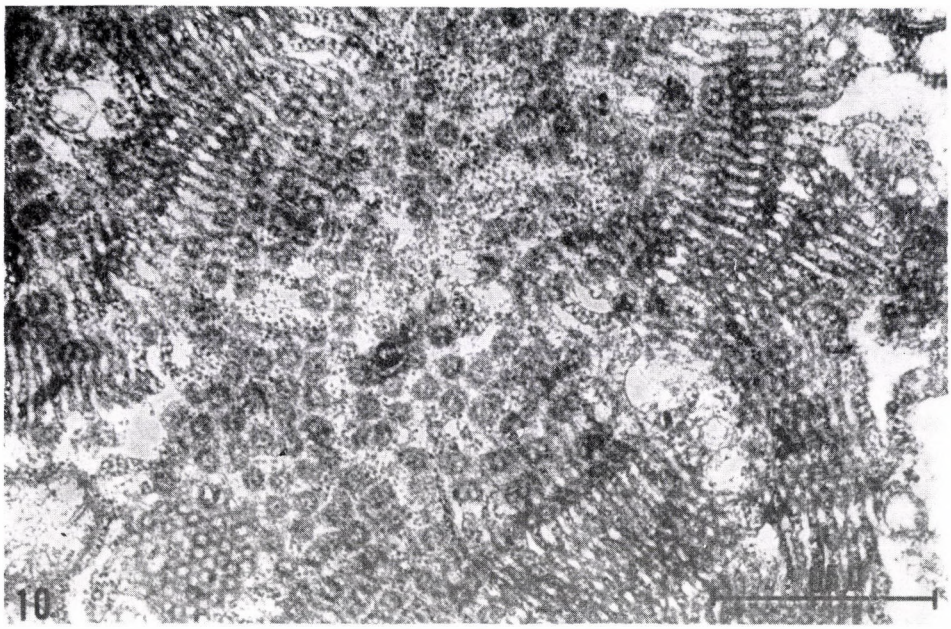

Fig. 10. KB cell 3 days after infection with type 12 adenovirus. Part of the highly organized membrane system filling the cytoplasm is shown. Osmium tetroxide fixation. Epon embedding. Uranyl acetate and Karnovsky staining. Magnification × 60 000

network formed in the nuclei of infected cells, and also for the analysis of virus particles and crystals.

In this way the structure of the individual virus particles appeared more clearly than after embedding in Epon. The particles contained a very electron dense central nucleoid surrounded by a clearer viroplasmic ring and a thin membrane with a sharp border.

By treatment with 0.5 per cent pepsin for 20 minutes, most of the protein of nuclei was digested. The chromatin was present in the form of highly electron dense amorphous clumps along the nuclear membrane. The viral nucleic acid was also resistant to pepsin treatment. The network or crystalline-like amorphous matter remained intact even after one hour pepsin treatment (Fig. 11).

Digestion with ribonuclease for one hour caused the disappearance of both the ribosomes from the cytoplasm and the RNA components from the nucleoli. No damage was demonstrable in the viral nucleic acid, in the electron dense reticular pattern or in the osmophilic matter forming amorphous masses (Fig. 12).

Particular attention has been devoted to the effects of desoxyribonuclease. Digestion with this enzyme for 1 to 2 hours already damaged some viruses within a crystalline array. The density of the nucleoids decreased or even the

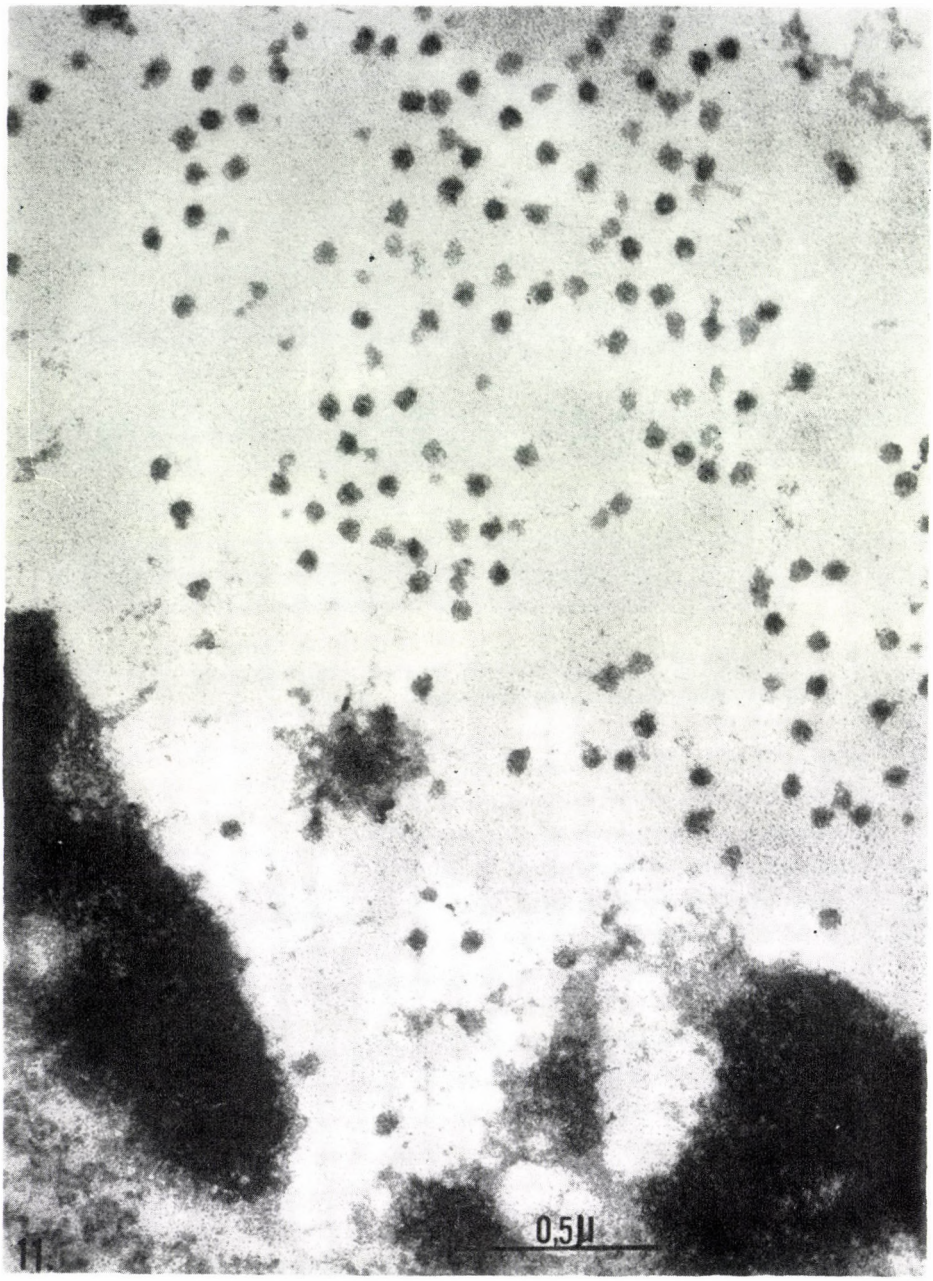

Fig. 11. KB cell 5 days after infection with type 12 adenovirus. The ultra-thin sections had been incubated in 0.5 per cent pepsin for 30 minutes. The nuclear protein was digested and the chromatin aggregated near the nuclear membrane. The central, electron dense substance of the viruses resisted this treatment. Formalin fixation. GMA embedding. Uranyl acetate and Karnovsky staining. Magnification ×60 000

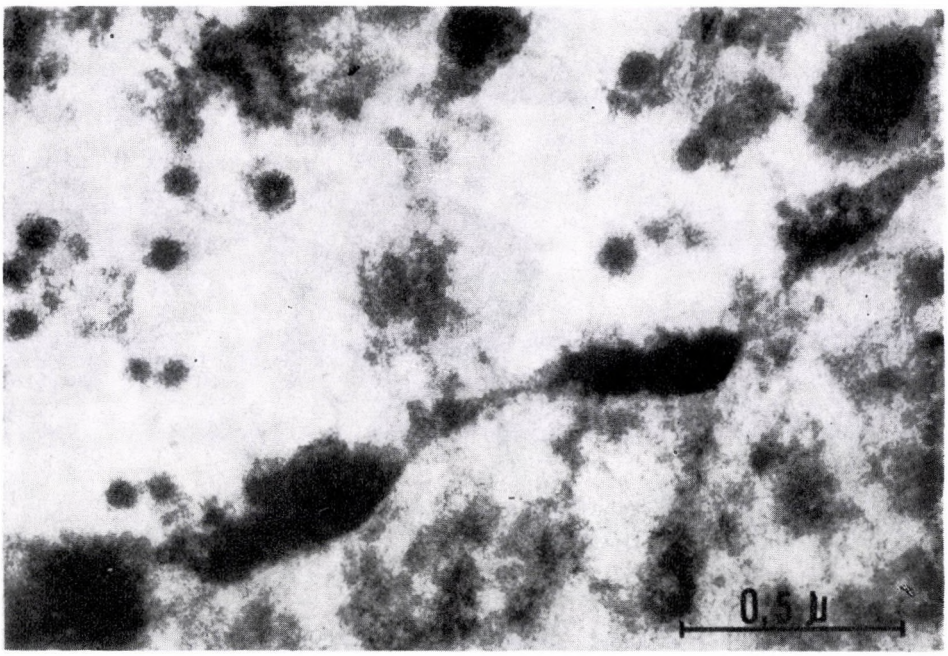

Fig. 12. KB cell five days after infection with type 12 adenovirus. Incubation with 0.1 per cent ribonuclease for one hour. Cytoplasmic RNA granules were digested, viral particles remained unchanged. Formalin fixation. GMA embedding. Uranyl acetate and Karnovsky staining. Magnification $\times 60\,000$

whole nucleoid disappeared in some particles. The effect was complete after 4 hours' incubation (Fig. 13). In preparations subjected to such treatment the nucleoids of all viral particles disappeared and only the thin proteinaceous membranes of the particles persisted. The amorphous matter forming the osmophilic reticular pattern resisted to prolonged desoxyribonuclease treatment.

Discussion

Recent progress in electron microscopy and the use of standard preparation methods have offered a firm basis for the exact morphological study of both normal and impaired cells. The aldehyde fixatives and embedding in water-soluble synthetic polymers [7, 8, 9] allow a direct histochemical characterization of the substances present in organelles or viruses within the cells. These methods have remarkably contributed to the information concerning a number of viruses.

Adenoviruses belong to the group of viral agents most extensively studied by both light and electron microscopy [10—32]. Type 12 adenovirus-infected cells were, however, scarcely examined at the ultrastructural level,

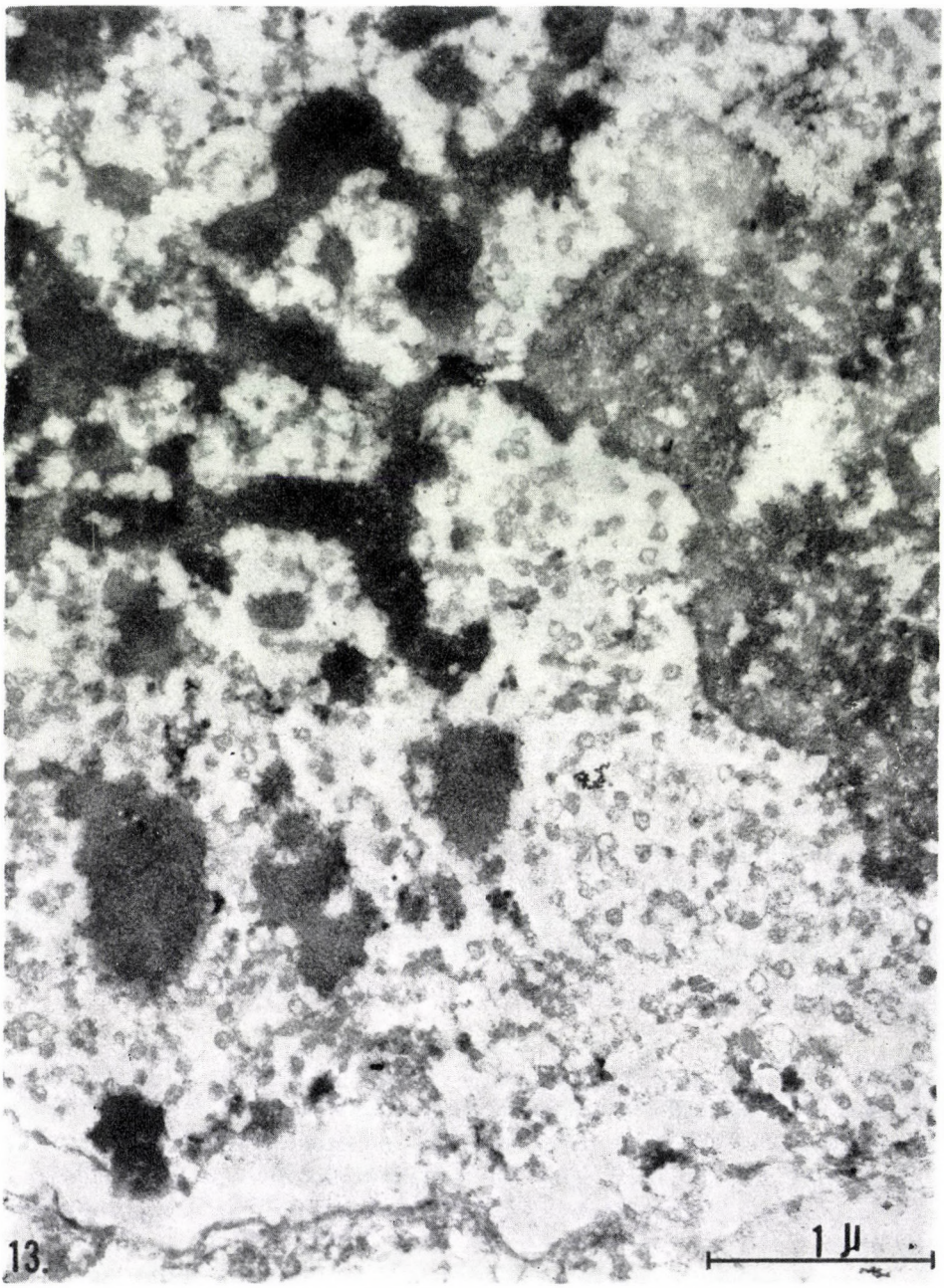

Fig. 13. KB cell 5 days after infection with type 12 adenovirus. Incubated in 0.1 per cent desoxyribonuclease for 4 hours at 37°C. The DNA containing nucleoids of the particles disappeared and only the ring-like protein shell remained intact. The network-forming electron dense substance in the nuclei resisted enzyme treatment. Formalin fixation. GMA embedding. Uranyl acetate Karnovsky staining. Magnification ×30 000

in spite of their tumour-inducing capacity having repeatedly been described in several animal species [2, 4, 5, 6].

According to our observations, the cellular damage induced by adenovirus type 12 remarkably resembled those produced by type 5 of the same group of agents [22, 23, 24, 25, 26]. The 4 phases of cytopathological events described for cells infected with type 5 adenoviruses by GODMAN *et al.* [27] could all be observed with type 12.

Before the appearance of viruses in the nucleus, a rapid accumulation of an osmophilic, highly electron dense substance takes place forming mostly a reticular pattern or occasionally a hexagonal crystalline array. This substance is resistant to both types of nucleases and to pepsin. It is therefore most probably a particular protein similar to that found in the acidophilic, Feulgen-negative inclusions not-fluorescing with acridine orange and developing after infection with type 5 adenovirus [16, 25, 26, 27, 32].

The above structure within the nucleus was gradually replaced by virus aggregates or crystals varying in size and number. Both aggregates and crystals were composed of a variable number of individual spheroid or oval particles 70 mμ in diameter. The particles had a sharp borderline, a moderately electron dense viroplasm and an innermost core of highly electron dense nucleoid. Treatment with ribonuclease had no effect on either of these structural components of the virus. All structural elements of the virus but the central electron dense part were destroyed by pepsin treatment. The latter was, however, sensitive to prolonged desoxyribonuclease digestion.

The increased DNA content of nuclei of adenovirus-infected cells has already been described [33]. Ultracentrifugal analysis of the components of cells infected with type 5 adenovirus have shown that the increased DNAcontent of the nucleus was a result of viral DNA accumulation [25].

In the present studies the high DNA content of the nucleoids of viruses inside the nuclei of infected cells was demonstrable *in situ*.

The submicroscopic cellular damage induced by type 12 adenovirus was essentially similar to that described for other types of the same group of agents [25, 26, 27, 34]. These lesions were, however, quite unspecific as they may be produced also by autolysis, immunolysis [35], and by some chemotherapeutics as well [34, 36, 37].

Hypertrophy of nucleoli was frequently observed. These structures often persisted also in nuclei stuffed with viruses, as observed in the case of some other adenoviruses [27, 34]. A rough granulation was often seen in the nucleoli of infected cells. This phenomenon has been described, however, also for the non-infected cells in cultures infected with type 5 adenovirus [27]. In our material there were also nucleolar lesions similar to those elicited by Actinomycin [38, 39] or Mytomycine-C [40]. We can offer no explanation for these morphological phenomena, which might represent some disturbance at the level of

DNA primed RNA synthesis. The preparations examined by us contained no lamellar structures in the nucleus similar to those found by others in cells infected with type 5 adenovirus [31].

In the cytoplasm of infected cells, only common degenerative lesions were present. Some cells, however, exhibited a highly organized lamellar structure in their cytoplasm. To our best knowledge, no similar findings have been reported up to now.

In some damaged cells virus aggregates were demonstrable also in the cytoplasm. On the basis of observations with type 5 adenovirus, this phenomenon is generally supposed to be a sign of grave degeneration, *e. g.* rupture of the cell membrane [27].

Efforts were made to discover lesions also in the eclipse phase. Most of our observations were, however, made on infected cells already carrying newly formed viral particles. The excellent review by BERNHARD [34] gives a survey on a number of recently developed methods (ferritin-conjugated antibodies, electronmicroscopic cytochemistry and autoradiography) and suggests new perspectives in the parallel study of morphological and metabolic events in virus-infected cells prior to the development of virus particles.

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PHAGE-TYPING OF ESCHERICHIA COLI O124 : K72(B17) BY THE EXAMINATION OF LYSOGENICITY

By

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Summary. By detecting temperate phages carried by *E. coli* O124: K72(B17) strains, a typing scheme for this organism has been elaborated. The cultures were obtained from water-borne outbreaks, multiple and sporadic cases of enteritis, and carriers. The 1526 strains isolated in various parts of Hungary were divided into 11 well-differentiated and stable types. An association has been revealed between phage type and the time of lactose fermentation. In western and northern parts of Hungary type 1, in south-eastern regions type 3 predominated. As confirmed by data obtained in various outbreaks, the typing method is suitable for epidemiological investigations.

Escherichia coli serogroup O124 : K72(B17) occupies a special position among enteropathogenic coli bacteria. While rarely associated with infantile enteritis, it frequently causes sporadic dysenteriform disease in children and adults and may be responsible for water and food-borne outbreaks.

Strains belonging to *E. coli* serogroup O124 : K72(B17) (hereinafter *E. coli* O124) were first isolated from sporadic cases by EWING and GRAVATTI in 1944 [1]. In 1949, HOBBS, THOMAS and TAYLOR [2] described a school outbreak of gastroenteritis caused by a slow-lactose-fermenting organism named paracolon 411. The authors mentioned that TAYLOR isolated 13 similar strains from food-poisoning occurring in a prisoner-of-war camp. EWING [3] in 1953 described that paracolon 411 was identical with *E. coli* O124 and possessed flagellar antigen H30. In addition to the Hungarian results, KLIMENKO [4] called attention to SUZUKI's work, who had isolated *E. coli* O124 from stools of patients with enteritis. MATSUMOTO and HARA [5] in 1959 reported on a new serotype, O124 : K72(B) : H12. EWING *et al.* [6] in a monograph published in 1963 described that their 39 *E. coli* O124 strains derived from 10 different countries belonged to 7 serotypes. In Rumania COSTIN [7] isolated *E. coli* O124 strains from children and adults with sporadic enteritis.

The occurrence of this organism in Hungary was first observed by KÉTYI, KNEFFEL and DOMJÁN [8] in connection with a water-borne outbreak in Szentgotthárd. In 1959 the water-borne epidemics in Veszprém and Parádsasvár were described by BORIÁN *et al.* [9] and LÁNYI *et al.* [10], respectively. A water-borne outbreak in Tatabánya, the occurrence of *E. coli* O124 in Komárom county and an attempt for phage typing of the organism were reported

by KOLTA and DEÁK in 1961 [11]. HANNY and HORVÁTH [12] described in 1963 a house outbreak of food poisoning due to *E. coli* O124.

According to the review of HABÁN [13], the number of *E. coli* O124 strains isolated in Hungary shows an increasing tendency. In certain years or certain regions the incidence of this organism reaches or even exceeds 50 per cent of Shigella strains; for example, in 1960 more *E. coli* O124 than Shigella strains were isolated in Baranya county.

KUBINYI [14, 15] has pointed out that in Hungary the incidence of enteritis is about 10 times higher than that of Shigella dysentery. His data indicate that about 5—6 per cent of enteric infections that occurred during the years 1956—1964, were due to *E. coli* O124. In absolute numbers this corresponds to an annual incidence of 8—10 thousand *E. coli* O124 infections. Within the above period *E. coli* O124 was involved in 10 epidemics: in 4 water-borne outbreaks it was the only pathogenic agent, in 4 other water-borne outbreaks it occurred with other pathogens and it caused 2 mass outbreaks of food poisoning.

Contact infections with *E. coli* O124 are presumably fairly frequent. During an institute-outbreak affecting 32 persons in Kecskemét, 3 contact infections were observed. According to KUBINYI [15], in the Veszprém epidemic 30 per cent of the cases occurred after sanitation of the infected water supply. These cases were therefore regarded as contact infections.

During the last five years in Komárom county KOLTA [16] found *E. coli* O124 on two or more repeated examinations in the faeces of 131 persons (17.4 per cent of all positive cases). Fifteen individuals carried this organism for more than one year.

In view of the frequent occurrence in Hungary of water and food-borne outbreaks, sporadic cases and symptomless carriers, it seemed desirable to make an attempt for improving epidemiological investigations by elaborating the phage typing of the organism in question.

This work has been carried out in the Phage Laboratory of our institute since 1961. Two different methods have been considered.

(1) From the faeces of patients and carriers, phages acting on *E. coli* O124 strains were isolated and adapted.

(2) Typing was attempted by detecting temperate phages liberated from *E. coli* O124 strains on indicator cultures.

Materials and methods

Materials. Faecal samples and 1526 *E. coli* O124 strains obtained from 1358 persons originated from larger and smaller outbreaks, sporadic cases and carriers scattered at various parts of Hungary. Some strains were received from DR. I. D. COSTIN, Timişoara, Rumania.

Adapted phages. By the method of EÖRSI, JABLONSZKY and MILCH [17], from the faeces of patients with enteritis 5, from sewage 3 phages were isolated. The phages were used undiluted or at the R. T. D.

Culture medium. Propagation of phages and liberation of temperate phages were performed in pH 7.2 Hartley broth. Typing was carried out on Hartley agar plates.

Indicator strains. For detecting temperate phages, non-lysogenic *E. coli* B and *E. coli* 36 cultures, *Shigella flexneri*, *Shigella sonnei phase II* and *E. coli* O124 strains isolated from our material were used.

Detection of lysogenicity. For the liberation of temperate phages the method of ANDERSON and FELIX [18] was modified as follows. The examined strain was inoculated into broth, shaken at 37°C for 4 hours, then centrifuged. The supernatant was heated to 58°C for 30 minutes, then dropped onto agar plates seeded with 2 hour cultures of the indicator strains. The plates were read after incubation overnight.

Results

(1) From 56 faecal specimens 12 phages acting on *E. coli* O124 were obtained. Three phages were isolated from sewage examined in connection with water-borne outbreaks.

Eight phages were selected and adapted to the corresponding strains. More detailed examinations showed that these phages were not specific, as, in addition to *E. coli* O124, they lysed some other *E. coli* and Shigella strains. With our set of phages 802 *E. coli* O124 cultures were examined. It was found that 60 per cent of the strains were uniformly lysed by all phages. Phage resistant cultures occurred in 10 per cent. The remaining 30 per cent of the cultures fell into 25 phage types. Thus typing with these phages was insufficient for epidemiological purposes. Two interesting observations will be discussed later.

(2) By determining the lytic spectrum of temperate phages released by lysogenic *E. coli* O124 strains, 11 well-differentiated stable types were established.

Table I

Typing scheme for E. coli O124 : K72(B17) on the basis of lytic activity of temperate phages

Phage type	Indicator strains					
	coli 36	SoR	L89	Dc 138	coli B	Fl 1b 262
1	+	−	−	−	−	−
1a	+	−	+	−	−	−
1b	+	−	−	+	−	−
2	+	+	−	−	−	−
2a	+	+	−	+	−	−
2b	+	+	−	−	+	−
3	−	+	−	−	−	−
3a	−	+	−	+	−	−
4	−	−	−	+	−	−
5	−	−	−	−	+	−
6	−	−	−	−	−	+

The lytic activity of temperate phages is presented in Table I. Each lytic spectrum represents one separate phage type.

The stability of *E. coli* O124 phage types *in vivo* and *in vitro* was confirmed by the following findings. Strains isolated from the same persons on repeated examinations belonged to the same types. Cultures maintained in the laboratory for years showed no alteration in phage type.

Table II shows the phage-type distribution of *E. coli* O124 strains. Among

Table II

Phage type distribution of E. coli O124 : K72(B17) strains

Phage type	No.	%	Phage type	No.	%
1	587	60.5	3	146	15.1
1a	7	0.7	3a	8	0.8
1b	15	1.5	4	52	5.4
2	64	6.7	5	2	0.2
2a	45	4.6	6	29	3.0
2b	15	1.5			

No. of typable strains, 970 (63.6%)
No. of untypable strains, 556 (36.4%)
Total number of strains, 1526

1526 strains examined during the last four years, 970 typable cultures occurred (64 per cent). Strains collected from various parts of the country belonged most frequently to type 1 and type 3 (60 and 15 per cent of typable strains).

The regional distribution of phage types of *E. coli* O124 strains is presented in Table III.

Table III

Regional distribution of typable E. coli O124 : K72(B17) strains

Area	No. of strains belonging to phage type						Total
	1	2	2a	3	4	Other	
Hungary West	455	36	18	90	43	57	699
Hungary North and Central	126	15	16	18	3	14	192
Hungary South-east	6	10	11	37	5	2	71
Rumania	—	3	—	1	1	3	8
Total	587	64	45	146	52	76	970

In western parts of Hungary type 1 predominated; other types such as 3 and 4 were frequently met with.

In northern and central Hungary, as compared to type 1, other types were rare. In south-eastern Hungary type 3 predominated.

In spite of the small number of strains, it is interesting to note that among cultures obtained from Rumania type 1 was absent and only types 2, 2b and 3 were encountered.

With the adapted phages two interesting observations have been made.

(i) In the examined material 25 *E. coli* O124 strains occurred which were lysed only by phage L81. This phage was isolated from the faeces of a patient with dysentery. These *E. coli* O124 cultures were uniform; they reacted only with phage L81, fermented lactose in 2 days, and carried no temperate phages active on any of our indicator strains. In some cases epidemiological connections were revealed among such *E. coli* O124 cultures.

(ii) Examination of 28 phage type 2a strains revealed that none of these cultures were sensitive to adapted phages.

It was interesting that while *E. coli* O124 strains isolated by various authors were generally uniform in biochemical properties, they differed in the time of lactose fermentation: HOBBS *et al.*, 4—21 days; EWING, 2 or more days; TAYLOR, 1—7 days; COSTIN 8—11 days; KÉTYI *et al.* 2 days; LÁNYI *et al.* 3—7 days; BORIÁN *et al.* 5—10 days.

Thus it was interesting to examine whether there was an association between phage type and the incubation time needed for positive lactose reaction. Results showing lactose fermentation by 491 strains are presented in Table IV. Most of the strains fermented lactose in 1 to 10 days, the majority

Table IV

Association between phage type and lactose reaction of E. coli O124 : K72(B17) strains

Phage type	No. of strains fermenting lactose in days					Total
	2—3	4—5	6—8	9—10	11	
1, 1a, 1b	11	38	197	39	6	291
2, 2a	46	2	—	—	—	48
2b	—	4	8	—	—	12
3, 3a	101	7	2	—	—	110
4	13	6	8	3	—	30
Total	171	57	215	42	6	491

between the 6th and 8th day. The number of positive tubes inoculated with *types 1, 1a and 1b* gradually increased till the 8th day, then decreased. *Types 2 and 2a* fermented that sugar in 2 or 3 days, while *type 2b* in 5 or 6 days. *Types 3 and 3a* were also positive in 2 or 3 days. No such regularity was observed

with *type 4* strains. Some prompt-lactose-fermenting strains also occurred; these cultures, however, were not lysogenic.

Only a few of the examined 1526 *E. coli* O124 strains were subjected to H antigen analysis. Thus no conclusions can be drawn as to the association between serotype and phage-type.

Discussion

Identification of bacteria on the basis of lysogenicity was first performed in 1932 by BURNET [19]. SCHOLTENS [20], examining phages carried by *S. paratyphi-B*, elaborated a typing scheme for this organism. A similar method for the typing of *S. typhi-murium* was described by BOYD [21]. This principle has since been applied successfully for other bacteria.

Typing of *E. coli* O124 with adapted phages did not yield the required results. Therefore the somewhat more laborious method based on lysogenicity had to be applied. A disadvantage of the latter was that the number of untypable strains was relatively high. This finding may be due to the considerable frequency of non-lysogenic strains; it may, however, be assumed that in reality these strains carried temperate phages which, in lack of suitable indicator strains, remained undetected. Finally, it is obvious that after a larger outbreak caused by an untypable *E. coli* O124, sporadic cases due to the same organism were frequently encountered. Thus the number of typable strains was highly decreased by the fact that one food-borne and two water-borne outbreaks were caused by untypable strains.

In other outbreaks typable strains were involved. Thus some strains originating from the Szentgotthárd water-borne outbreak [8] belonged to type 2. Strains isolated from the water-borne outbreak at Parádsasvár [10] fell into type 1. Water-borne outbreaks that occurred in Szeged and Marcali in 1964 were associated with type 3.

No predominance of either type was noted among sporadic cases and carriers. It can therefore be concluded that any *E. coli* O124 phage type may be responsible for epidemics and sporadic infections and occur in symptomless carriers.

Typing of *E. coli* O124 by examining the lysogenicity of strains offers valuable help in the investigation of epidemiological problems and in the detection of infective sources. On several occasions it was revealed by phage typing that a seemingly uniform epidemic had started from more than one source. Thus in a children's home the continuous occurrence of *E. coli* O124 enteric infections was noted between August and December, 1963. The first cases affecting 3 members of the staff were due to type 3. Till September 20, the same phage type was isolated from 7 children. At the end of August, one child

harboured an untypable strain; such untypable cultures were detected till December 20 in 6 faecal specimens from 4 children. Type 1 appeared first on September 11; till December 20 it was isolated from 12 specimens from 3 children. Therefore, in a seemingly uniform epidemic phage typing revealed the involvement of at least three different infective sources. On repeated examinations two children excreted two different types; this observation indicated that after successful antibiotic therapy these children were reinfected from the environment with other *E. coli* O124 phage types.

Our study is far from complete. In addition to improving the method, we desire to examine the association between flagellar antigens, biotype, and phage type of *E. coli* O124 : K72(B17) strains.

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INVESTIGATIONS INTO THE OLIGOSACCHARIDE DECOMPOSITION BY CANDIDA BRUMPTII (LANGERON ET GUERRA) LANGERON ET GUERRA AND PROCANDIDA GRUBYI NOVÁK ET VITÉZ

By

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Summary. It has been shown that *Candida brumptii* and *Procandida grubyi* decompose sucrose and maltose by intracellular enzymes. The maltose-splitting enzyme of both species and the sucrose-splitting enzyme of *P. grubyi* are identical with enzymes revealed in other yeasts. The sucrose-splitting enzyme of *C. brumptii* represents a new, acetone-resistant type. Neither of the two species produced invertase.

The difference between the sugar assimilation and sugar fermentation spectra of the examined organisms is due to a difference in their sugar transportation systems.

In comparing the carbohydrate decomposing activity of yeasts we have investigated the association between the splitting of sucrose and maltose and the enzymes concerned. In view of results obtained with *Procandida stellatoidea*, syn. *Candida stellatoidea* [6, 9], it seemed desirable to examine some yeasts which differ as to sugar utilization spectrum from this organism only in one property. As the spectrum of *P. stellatoidea* can be expressed as DGM-dm*, we intended to examine one yeast species with DGM-d and another with DSM-dm utilization spectrum. The former properties were shown by *C. brumptii* the latter by the recently recognized *P. grubyi* [8]. Sucrose, maltose and raffinose utilization and splitting by the two species have therefore been examined.

Materials and methods

Strains *C. brumptii* 229/1961 and *P. grubyi* 469/1961 have been maintained in our collection (S. I. H.). The cultures were grown on Csillag's molasses agar [1] in Roux bottles. Live and acetone-treated cells and cell-free extracts were prepared and chromatographic examinations were performed as described in references 3,4 and 6.

Results

Candida brumptii

Raffinose was not decomposed by any of the preparations and was not taken up by live cells (Figs 1—3).

* Abbreviations [12]: D or d = glucose, G or g = galactose, S or s = sucrose, M or m = maltose. Capitals stand for assimilation, small letters for fermentation. Only positive reactions are indicated.

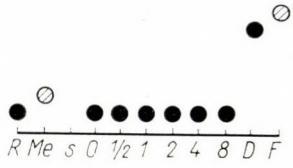


Fig. 1

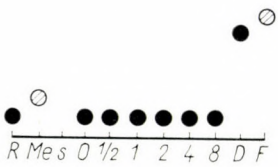


Fig. 2

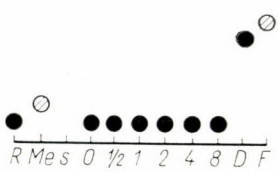


Fig. 3

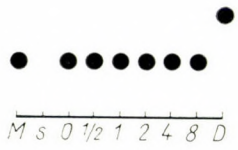


Fig. 4

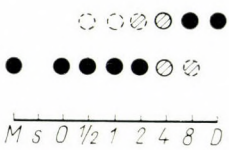


Fig. 5

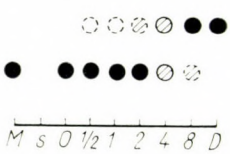


Fig. 6

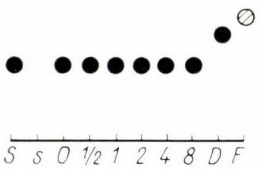


Fig. 7

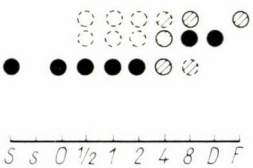


Fig. 8

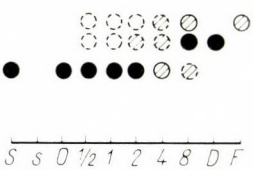


Fig. 9

Fig. 1. Raffinose utilization by intact *C. brumptii* cells. 750 mg live wet cells and 60 mg raffinose in pH 7.2 *M*/30 phosphate buffer in 3 ml volume. Copy of the chromatogram. Left raffinose, melibiose and suspension, right glucose and fructose controls. Numbers under the start line represent the sampling intervals in hours

Fig. 2. Raffinose utilization by acetone treated *C. brumptii* cells. 750 mg acetone treated liv[e] wet cells; others as indicated in Fig. 1

Fig. 3. Raffinose utilization by cell-free extract of *C. brumptii* cells. Cell-free extract of 600 mg quartz sand-disintegrated live wet cells; others as indicated in Fig. 1

Fig. 4. Maltose utilization by intact *C. brumptii* cells. 750 mg live wet cells and 60 mg maltose in pH 7.2 *M*/30 phosphate buffer in 3 ml volume. Copy of the chromatogram. Left maltose and suspension, right glucose controls. Numbers under the start line represent the sampling intervals in hours.

Fig. 5. Maltose utilization by acetone treated *C. brumptii* cells. 750 mg acetone treated live wet cells, others as indicated in Fig. 4

Fig. 6. Maltose utilization by cell-free extract of *C. brumptii* cells. Cell-free extract of 600 mg quartz sand-disintegrated live wet cells, others as indicated in Fig. 4

Fig. 7. Sucrose utilization by intact *C. brumptii* cells. 750 mg live wet cells and 60 mg sucrose in pH 7.2 *M*/30 phosphate buffer in 3 ml volume. Copy of the chromatogram. Left sucrose and suspension, right glucose and fructose controls. Numbers under the start line represent the sampling intervals in hours

Fig. 8. Sucrose utilization by acetone treated *C. brumptii* cells. 750 mg acetone treated live wet cells; others as indicated in Fig. 7

Fig. 9. Sucrose utilization by cell-free extract of *C. brumptii* cells. Cell-free extract of 600 mg quartz sand-disintegrated live wet cells; others as indicated in Fig. 7

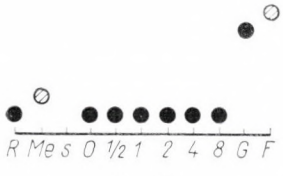


Fig. 10.

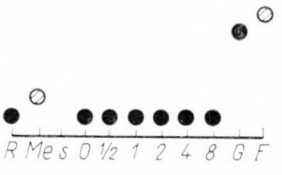


Fig. 11.

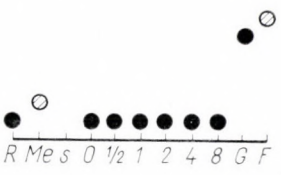
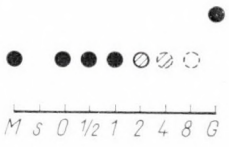


Fig. 12.



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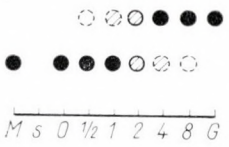

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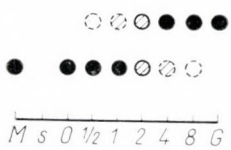

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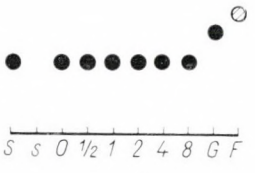

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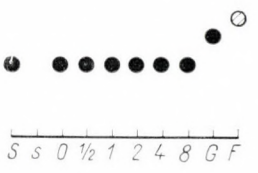

Fig. 17.

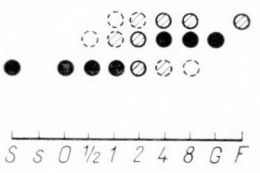

Fig. 18.

Fig. 10. Raffinose utilization by intact *P. grubyi* cells. 560 mg live wet cells and 60 mg raffinose in pH 7.2 *M*/30 phosphate buffer in 3 ml volume. Copy of the chromatogram. Left raffinose, melibiose and suspension, right glucose and fructose controls. Numbers under the start line represent the sampling intervals in hours

Fig. 11. Raffinose utilization by acetone treated *P. grubyi* cells. 740 mg acetone treated live wet cells, others as indicated in Fig. 10

Fig. 12. Raffinose utilization by cell-free extract of *P. grubyi* cells. Cell-free extract of 680 mg quartz sand-disintegrated live wet cells; others as indicated in Fig. 10

Fig. 13. Maltose utilization by intact *P. grubyi* cells. 560 mg live wet cells and 60 mg maltose in pH 7.2 *M*/30 phosphate buffer in 3 ml volume. Copy of the chromatogram. Left maltose and suspension, right glucose control. Numbers under the start line represent the sampling intervals in hours

Fig. 14. Maltose utilization by acetone treated *P. grubyi* cells. 740 mg acetone treated live wet cells; others as indicated in Fig. 13

Fig. 15. Maltose utilization by cell-free extract of *P. grubyi* cells. Cell-free extract of 680 mg quartz sand-disintegrated live wet cells; others as indicated in Fig. 13

Fig. 16. Sucrose utilization by intact *P. grubyi* cells. 560 mg live wet cells and 60 mg sucrose in pH 7.2 M/30 phosphate buffer in 3 ml volume. Copy of the chromatogram. Left sucrose and suspension, right glucose and fructose controls. Numbers under the start line represent the sampling intervals in hours

Fig. 17. Sucrose utilization by acetone treated *P. grubyi* cells. 740 mg acetone treated live wet cells; others as indicated in Fig. 16

Fig. 18. Sucrose utilization by cell-free extract of *P. grubyi* cells. Cell-free extract of 680 mg quartz sand-disintegrated live wet cells; others as indicated in Fig. 16

Maltose was not taken up nor decomposed by live cells (Fig. 4). Acetonized cells and cell-free extracts hydrolysed this sugar into glucose (Figs 5 and 6).

Sucrose was neither taken up nor split by live cells (Fig. 7). Acetone treated cells and the cell-free homogenate produced glucose and fructose from sucrose (Figs 8 and 9).

Procandida grubyi

Raffinose was neither taken up nor split by live cells or the preparations (Figs 10—12).

Maltose was incorporated by live cells; acetonized cells and the cell-free extract split this sugar into glucose (Figs 13—15).

Sucrose was not taken up by live cells; it was decomposed only by the cell-free extract (Figs 16—18).

Discussion

As shown by our results, *C. brumptii* produces both maltose and sucrose decomposing enzymes. The enzyme acting upon maltose belongs to the intracellular acetone-resistant type described earlier [5, 7]. The maltose-splitting enzyme of *P. grubyi* also belongs to this group (see below). In contrast, the sucrose-splitting enzyme of *C. brumptii* differs from the extracellular acetone-resistant (invertase, beta-h-fructosidase) and intracellular acetone-sensitive enzymes observed in our previous experiments. This sucrose-splitting enzyme is not identical with invertase, as it is intracellular and the cell-free extract containing the enzyme is inactive on raffinose. Neither is it identical with the other sucrose-splitting enzymes, since it is resistant to acetone (acetonized cells also decomposed the sugar). Thus taking into consideration that the acetone-sensitive group of sucrases contains two types — the first have been described by LINDEGREN *et al.* [2] and the second by NOVÁK and ZSOLT [11] — the sucrose-splitting enzyme of *C. brumptii* represents a new, fourth type among yeast "sucrases".

In view of the presence of a sucrose-splitting enzyme and the absence of sucrose assimilation and fermentation, it may be concluded that *C. brumptii* possesses neither aerobic nor anaerobic sucrase transportase enzyme systems. The presence of maltose assimilation and maltose decomposing enzyme and the absence of maltose fermentation indicate that this organism produces only aerobic maltose transportase.

It should be noted that *C. brumptii* was inadequate for a model that acts upon maltose but not on sucrose in cell-free extract. The same was true for *P. stellatoidea*, which, however, decomposed sucrose less actively [7]. Although sucrose-splitting can be eliminated by acetone or other lipid solvents

[5, 10, 11], except in the case of *C. brumptii*, it would be interesting to study a system exerting a pure maltose-decomposing activity.

P. grubyi also contains sucrose and maltose-splitting enzymes. The former corresponds to an intracellular, acetone sensitive "sucrase", the latter to an intracellular acetone resistant "maltase". The same types of enzyme were isolated from *C. solani* [5], *P. albicans* [10, 11] and *P. stellatoidea* [7]; the same type of "maltase" occurred in *Rhodotorula mucilaginosa* [13] and the same type of "sucrase" in *C. requinyii* [6]. As shown above, the "maltase" of *C. brumptii* also belongs to this type.

As sucrose is assimilated but not fermented by *P. grubyi*, it may be concluded that this organism produces aerobic but no anaerobic sucrose transportase. Transportation of maltose takes presumably place both aerobically and anaerobically, as the organism assimilates and ferments maltose.

It is interesting to note that in our experiments the uptake of only those sugars have been demonstrated which, as shown by diagnostic tests, are fermented under anaerobic conditions by the corresponding organism (maltose uptake by *P. grubyi*). The same results were obtained for *C. solani* [5], *P. albicans* [10] and *P. stellatoidea* [7]. Observations indicating that non-fermented but assimilated sugars were not transported, also confirm this conception. It may therefore be concluded that our incubation technique (resting incubation for 6—8 hours in narrow tubes containing 500—1000 mg wet cells in 3 ml 2 per cent sugar solution) provides anaerobic rather than aerobic conditions and is thus suitable for further examination of anaerobic transportation processes. At the same time it is obvious that in glucose-fermenting organisms the strictly substrate-specific nature of oligosaccharide transportase systems is responsible for the failure of fermentation of assimilated oligosaccharides.

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OCCURRENCE OF ATYPICAL MYCOBACTERIA IN MACACUS RHESUS

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Summary. (i) Fifty strains of atypical mycobacteria have been isolated from 33 animals out of 66 Macacus rhesus and 3 Cercopithecus monkeys. Of the strains 46 were dysgonic and multiplied only at 37°C. Four strains grew rapidly.

(ii) On the basis of photochromogenicity, dysgonic growth, enzyme activity and mouse virulence, 9 strains were identified as belonging to a new species. The recommended name for these bacteria is *Mycobacterium simiae.*

(iii) Of 16 non-chromogenic strains 6 were examined. As judged by their enzyme reactions, 5 of the cultures belonged to the avium group. In amidase spectrum 4 scotochromogenic cultures corresponded to human scotochromogenic strains. Among 4 rapidly growing cultures 2 saprophytic and 2 highly mouse-virulent organisms were distinguished.

(iv) The ability of the isolated mycobacteria to grow in mice makes probable that they may also produce chronic infection in monkeys. Pathogenicity to monkeys of the examined strains was not demonstrated.

In connection with the origin and classification of human atypical mycobacteria a number of problems await elucidation. RUNYON's classification [4] based on empirical and easily detectable properties, the time of growth and pigment production, can be very adequately used as a starting point in such examinations. Intensive studies have been performed on the occurrence of atypical mycobacteria in the environment of man and on the role of the patient in the transmission of the infection. In diseases caused by *M. kansasii* neither man-to-man infections, nor environmental sources have yet been revealed. The occurrence of atypical mycobacteria in sources other than man has been mentioned in a few reports. SMITH, KOVÁCS and HARRIS [5] in West Australia isolated 82 avian group Battey strains from 412 pigs. They found the same strains in milk, soil and dust. KUBICA, BEAM and PALMER [2] isolated 204 strains from soil and water. Among 80 strains by these authors 2 photochromogenic, 12 scotochromogenic, 22 non-pigment-producing and 29 rapidly growing cultures were distinguished. Biochemical, pathogenic and other properties of the strains have not been discussed in detail. CHAPMAN, BERNARD and SPEIGHT [1] isolated 261 strains at an incidence of 33.9 per cent from milk samples; 5 strains were isolated directly from the udder. Among the isolates 12 photochromogenic cultures occurred which, because of their different enzyme reactions and antigenic properties, could not be identified as *M. kansasii.* MALLMANN, MALMANN and ROBINSON [3] studied 40 atypical

mycobacterial strains isolated from cattle and pigs. Ten strains showing atypical photochromogenicity were described as pseudochromogens, 12 strains belonged to the avium group and 4 strains represented rapidly growing mycobacteria.

To our knowledge, we have been the first to report on the occurrence of attenuated tubercle bacilli and atypical mycobacteria in monkeys imported for research purposes [6]. These observations have made necessary to examine spontaneous mycobacterial infections in monkeys. The great number of monkeys imported by the State Institute of Hygiene, Budapest, for the preparation of tissue cultures provided an ample material for this study.

Materials and methods

The organs of healthy, non-tuberculotic animals including 66 Macacus rhesus and 3 Cercopithecus monkeys 1 to 2 years of age, weighing 1.5 to 2 kg, were examined. In the first period of examinations 47 animals were studied. In these experiments axillary, inguinal and mesenterial lymph nodes were worked up together; separate cultures were made from the liver, spleen and lungs. In subsequent investigations the lymph nodes of 22 monkeys were cultured separately after homogenization in a teflon homogenizer. The latter technique gave much better results. After homogenization the organs were treated with 6 per cent sulphuric acid. After centrifugation the sulphuric acid remaining in the deposit was washed up with sterile phosphate buffer. The deposit was then resuspended in a solution containing 2 to 5 per cent bovine albumin and metabolites of the Krebs-Szent-Györgyi cycle. Each material was inoculated onto 4 Löwenstein—Jensen medium slants and into 2 tubes of liquid haemolysed rabbit blood medium. After 1 and 2 months' incubation the latter cultures were centrifuged and the deposit was seeded on Löwenstein—Jensen medium. Observation lasted for 3 months. The isolates were examined for growth on Löwenstein—Jensen, agar and broth media at room temperature and at 37° C. Pigment production was examined in dark and after lighting. Part of the strains were examined for biochemical reactions as described by BÖNICKE: catalase, peroxidase, nitrate reductase, niacin, acetamidase, benzamidase, nicotinamidase, urease, pyrazineamidase, succinamidase and allantoinase. In addition, lipase activity against Tween 80 was determined. Sensitivity to streptomycin, isoniazid and PAS (1, 10 and 50 μg concentration per ml), was examined on Löwenstein—Jensen medium using 0.2 mg inocula. Pathogenicity of the cultures was examined by intravenous injection of 1 mg bacteria into 10 white mice in each case. Recovery of the organisms from the animals was attempted 30 and 60 days after injection. The number of colonies isolated from the spleen and liver is indicated by — to +++ marks.

Results

Mycobacteria were cultured from lymph nodes and organs of 33 out of 69 monkeys. The total number of isolated strains was 50 (Table I). Nine strains were recovered only by enrichment in haemolysed blood medium. Accordingly, this method increased the number of isolated strains by 37.5 per cent.

Monkeys falling into group I were positive in 36 per cent, while animals of group II nearly in 73 per cent. The number of strains isolated from visceral organs was considerably higher in group II. This was presumably due to the fact that treatment in the teflon homogenizer very effectively liberates bacteria from the tissues. In this group mycobacteria were isolated in 9 monkeys only from lymph nodes, in 5 monkeys from lymph nodes and viscera and in 2

Table I

Occurrence of mycobacteria in monkeys

Monkeys examined		Monkeys positive for mycobacteria		Organs positive for mycobacteria		Total No. of strains
Group	Number	Number	%	Lymph nodes a b c	Viscera	
I	47	17	36	16	1 (2.1%)	17
II	22	16	73	9 9 8	7 (31.8%)	33
Total	69	33	48	42	8	50

Lymph gland: a = inguinal, b = axillary, c = mesenterial

monkeys only from viscera. One lymph node was positive in 6, two in 4, and three in 4 animals. Microscopic examination indicated that most strains consisted of very short acid fast rods or granules.

Table II

Examination of mycobacterial strains isolated from monkeys

RUNYON's group	Number of strains investigated	Number of strains examined	
		biochemically	in vivo
I	9	9	9
II	4	4	4
III	16	6	7
IV	4	4	4
Total	33	23	24

Table II shows our strains according to RUNYON's classification. Biochemical reactions were performed with 23 strains; 24 strains were inoculated into mice. Among 33 strains (one from each monkey), 9 photochromogenic, 4 scotochromogenic, 16 non-chromogenic and 4 rapidly growing cultures were encountered.

Properties of photochromogenic strains (Table III). The majority of these organisms exhibited dysgonic growth on Löwenstein—Jensen medium; they grew poorly even after 2—3 weeks. No growth was observed on agar or in broth. Changes of the photochromogenic property were observed in a few cases. Four strains produced nitrate reductase. Two acetamidase and 3 urease positive cultures were revealed. Four strains were negative for all amidases. Resistance testing have the following results (Table IV). Three strains were sensitive to 50 μg INH but only partly sensitive to 10 μg INH. With the exception of

Table III

Biochemical properties of photochromogenic mycobacteria

Designation of strain	Character of culture	Niacin	Nitrate red.	Catalase	Peroxidase	Lipase	Acetamidase	Benzamidase	Urease	Nicotinamidase	Pyrazinamidase	Succinamidase	Allantoinamidase
No. 5	dysgonic S-var.	−	+	++	+	+	−	−	+	+	−	−	−
No. 14	S-var.	−	++	++	+	−	−	−	+	±	−	+	−
No. 20	S-var.	−	−	++	++	±	−	−	−	−	−	−	−
No. 25	dysgonic S-var	−	+	++	+	−	+	−	−	+	+	−	+
No. 27	dysgonic S-var.	−	−	++	++	+	−	−	−	−	+	−	−
No. 29	dysgonic R-var	−	++	++	++	+	+	−	+	±	−	−	−
No. 52	S-var	−	−	++	++	+	−	−	−	−	−	−	−
No. 61	S-var.	−	−	++	+	+	−	−	−	−	−	−	−
No. 68	S−R	−	−	++	++	+	−	−	−	−	−	−	−

Table IV

Drug resistance of mycobacterial strains isolated from monkeys

Group	Designation of strains	Isoniazid, μg			Streptomycin, μg			PAS, μg		
		1	10	50	1	10	50	1	10	50
I	No. 5	+	+	±	+	−	−	+	−	−
	No. 14	+	±	−	+	+	+	+	+	+
	No. 20	+	±	−	+	+	±	+	+	+
	No. 25	+	+	±	+	+	+	+	+	+
	No. 27	+	+	+	+	+	+	+	+	+
	No. 29	+	+	±	+	+	+	+	+	+
	No. 52	+	±	−	+	+	±	+	+	+
	No. 68	+	+	±	+	+	−	+	+	±
	No. 61	+	+	+	+	+	±	+	+	−
II	No. 32	+	+	±	+	±	−	+	+	+
	No. 47	+	+	−	+	+	±	+	+	−
	No. 51	+	±	−	+	+	+	+	+	+
	No. 64	+	+	+	+	+	+	+	+	+
III	No. 3	+	±	−	+	+	−	+	+	+
	No. 23	+	+	+	+	+	+	+	+	+
	No. 46	+	±	−	+	±	±	+	+	±
	No. 53	+	±	±	+	+	+	+	+	+
	No. 54	+	−	−	+	+	+	+	+	−
	No. 63	+	±	−	+	+	+	+	+	+
IV	No. 1	+	±	−	+	±	−	+	+	+
	No. 4	+	+	+	+	+	+	+	+	+
	No. 15	+	+	+	+	+	+	+	+	+
	No. 55	+	+	+	+	−	−	+	+	+

strain No. 5, all cultures were resistant to streptomycin and PAS. In animals (Table V) all strains multiplied intensively and were cultured in large numbers from the organs. Their mouse virulence except the strains No. 29 and No. 52 was low, as none of the animals from the other groups died within 90 days. In the lung of mice sacrificed after the observation period, several tubercles were revealed.

Table V

Mouse experiments with atypical mycobacteria isolated from monkeys

Group	Strains	Cultures from mice		Pathological changes in organism of mice					Dead after inoculation (in days)
		Days after inoculat.	Number of colonies	day	lung	spleen	kidney	liver	
I.	No. 5	65	+++	65	−	−	−	−	−
	No. 14	27	++++	75	++	−	−	−	−
	No. 20	65	++++	65	++++	+	+	±	−
	No. 25	90	+++	90	+++	−	−	−	−
	No. 27	90	++++	90	+++	−	−	−	−
	No. 29			34	±	++	++	−	9, 28, 34, 34, 34
	No. 52	63	++++	60	++++	−	−	−	34, 34
	No. 61	61	+++	84	±	+	−	−	−
	No. 68	73	++++	73	+	+	±	−	−
II.	No. 32	65	++	65	±	−	−	−	−
	No. 47	65	+++	61	±	+	−	−	22
	No. 51	60	++++	60	+++	+	−	−	22, 34, 36
	No. 64	52	++	52	−	−	−	−	6, 11, 20 32, 40, 52
III.	No. 3	53	++	90	−	+++	−	++	11, 22, 51, 63.
	No. 23	63	+++	63	−	+	++++	−	−
	No. 46	57	++++	70	−	+	−	−	9, 14, 19, 63, 67
	No. 53	33	++++	46	−	++	±	−	−
	No. 54	33	++	46	−	+	−	−	−
	No. 59	50	++++	35	+++	+	+++	++++	10, 13, 19, 31, 34, 46
	No. 63	70	++++	70	−	+	+++	−	4, 7, 8
IV.	No. 1	90	+	90	−	−	−	−	−
	No. 4	90	++++	90	−	−	++++	−	22, 33, 39, 56, 90
	No. 15	51	+++	53	−	−	++++	−	13, 14, 18 20, 20, 21
	No. 55	77	++++	77	−	−	−	−	−

Properties of scotochromogenic strains (Table VI). Of 4 scotochromogenic strains 1 produced nitrate reductase. Of the amidase enzymes urease was formed only by 2 strains. Of these strains two (No. 47, No. 51) were sensitive

to 50 μg INH. Strain No. 51 was sensitive to 50 μg streptomycin. Out of four strains inoculated into mice, two were virulent. After 60 days definite lesions were observed in the lung of mice inoculated with the strain No. 51. Of 9 mice, 3 died after 22, 34 and 36 days, respectively.

Table VI

Biochemical properties of scotochromogenic and rapidly growing mycobacteria

	Designation of strain	Character of culture	Niacin	Nitrate red.	Catalase	Peroxidase	Lipase	Acetamidase	Benzamidase	Urease	Nicotinamidase	Pyrazinamidase	Succinamidase	Allantoinamidase
Scotochromogenic	No. 32	S-var.	—	—	+	+	+	—	—	—	±	—	—	—
	No. 47	dysgonic S-var	—	—	±	—	—	—	—	—	—	—		—
	No. 51	S-var.	—	—	+	+	—	—	—	+	—	—	—	—
	No. 64	dysgonic S-var.	—	++	+	+	+	—	—	+	—	—	—	—
Rapidly growing	No. 1	Yellow mucoid	—	+	+	+	+	—	+	+	+	—	—	+
	No. 4	White S-var.	—	—	+	+	—	+	+	—	+	++	—	+
	No. 15	White S-var.	—	—	+	+	—	+	+	—	+	+	+	+
	No. 55	Yellow S-var.	—	+	+	+	+	—	—	+	+	+	—	—

Properties of non-chromogenic strains. Growth of these strains, especially in the first subculture, was very slow, dysgonic. Some cultures produced light creamy-yellow colonies. Biochemical reactions of 6 strains are presented in Table VII. Two strains produced nitrate reductase. With the exception of strain No. 53, in amidase spectrum the strains corresponded to the avium group. A slight urease activity was revealed in 4 strains. To 10 μg INH one strain was resistant, 4 moderately sensitive and one strain (No. 54) was sensitive. All strains were resistant to 10 μg streptomycin and PAS. When inoculated into mice, the examined strains multiplied intensively and were recovered in large numbers from spleen and liver. Three strains out of 7 caused death after 9 to 63 days. Post mortem severe changes were revealed in the kidneys and spleen.

Properties of 4 rapidly growing strains (Table VI). Strains No. 1 and No. 55 grew abundantly on agar and in broth after 4—5 days. Both strains exerted a wide spectrum amidase activity and produced nitrate reductase. The strain No. 55 was sensitive to 10 μg streptomycin and resistant to INH and PAS. Neither of the two strains was pathogenic to mice. In mice, strain No. 1 multiplied slowly, strain No. 55 intensively. These findings are indicative of the saprophytic nature of these strains. The other 2 quickly growing strains (No. 4 and No. 15) deserve special attention. On first culture these organisms grew slowly, but after some subcultures they multiplied rapidly even at room

Table VII

Biochemical properties of non-chromogenic mycobacteria

Designation of strain	Character of culture	Niacin	Nitrate red.	Catalase	Lipase	Peroxidase	Acetamidase	Benzamidase	Urease	Nicotinamidase	Pyrazinamidase	Succinamidase	Allantoinamidase
No. 3	S-var.	−	−	+	−	+	−	−	−	+	+	−	−
No. 23	S-var.	−	++	+	+	+	−	−	±	+	+	−	−
No. 46	S-var.	−	−	±	−	+	−	−	−	+	+	−	−
No. 53	S-var.	−	−	+	−	+	−	−	+	−	−	−	−
No. 54	dysgonic S-var	−	−	+	±	+	−	−	±	±	+	−	−
No. 63	dysgonic R-var.	−	++	+	+	+	−	−	±	+	±	−	−

temperature. On the basis of biochemical reactions (negative nitrate reductase, positive against 7-8 acylamides) they did not belong to the avium group. Both strains were resistant to 50 μg INH, streptomycin and PAS. They were highly virulent for mice; the infected animals died after 14—39 days. At autopsy severe lesions containing large numbers of acid fast bacteria were revealed in the kidneys.

Discussion

Our examinations have shown that atypical mycobacteria occur with considerably frequency (46 per cent) in Macacus rhesus monkeys. The organisms were isolated mainly from subcutaneous lymph gland, but in 8 cases also from spleen and liver. This makes it probable that the penetration of the microbes occurred through the skin. The majority of the isolated cultures was resistant to streptomycin and thus it may be assumed that they are able to multiply when transferred with kidney cells into tissue cultures. It is a question whether the strains are identical with known mycobacteria. As indicated by their enzyme spectrum and mouse pathogenicity, the non-chromogenic cultures probably belong to the avium group. KUBICA *et al.* [2] and KOVÁCS [7] have shown the wide occurrence in the environment of man of such strains and their capability of multiplying as facultative pathogens in domestic animals. Our observations revealed that non-domesticated animals such as monkeys may also carry these organisms.

The presence of photochromogenic strains in monkeys is of a certain significance. It is known that only *M. kansasii* and *M. balnei* are photochromogenic. Recently, CHAPMAN has described other photochromogenic mycobacteria. Our photochromogenic strains exhibited dysgonic growth and enzyme reactions differing from those given by *M. kansasii* or *M. balnei*. Though they

multiplied intensively in mice, their mouse virulence was low. These properties distinguish our cultures from the known photochromogenic mycobacteria, and as the organisms isolated by us correspond to new mycobacteria prevalent in monkeys, we recommend *Mycobacterium simiae* for their taxonomic name.

The 4 pigment-producing scotochromogenic strains, similarly to human pigment-producing cultures, are presumably variants of non-pigmented strains. The close relationship of the two saprophytic strains (No. 1 and No. 55) with *M. phlei* and *M. smegmatis* is probable. It is interesting that in some monkeys occurred simultaneously two strains differring in pigment production or biochemical reactions. Indicating the importance of the variability of mycobacteria, on subcultures strains No. 27 and No. 47 split off new variants. Strains No. 4 and No. 15 may derive from the avium group, but may as well represent new mycobacteria. Our results make necessary an antigenic analysis of the strains, in order to elucidate their relationship to known mycobacteria; we have shown that non-tuberculous mycobacterial infections may widely occur among monkeys living in Asian jungles. The fact that mycobacteria have been shown in one of three African Cercopithecus monkeys indicate that surveys should be performed also among these animals.

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BREAKDOWN OF AMINO ACIDS BY ENTEROBACTERIACEAE

IV. GLYCINE DECOMPOSITION TEST

By

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Summary. Simple routine tests based on alkalization and ninhydrin reactions have been described for determining the bacterial breakdown of glycine. By use of the method Enterobacteriaceae can be divided into glycine negative (Shigella, *S. paratyphi A*) and glycine positive (*E. coli*, Klebsiella, Citrobacter, Enterobacter, Serratia, Arizona, different salmonellae, Proteus-Providencia) groups. Among glycine positive genera (species) some minus variants occurred. When the microtest is applied instead of the macrotest, the observation period of one week is shortened to one day.

The present paper reports on our first experiments on simple, routinely applicable tests using the alkalization and ninhydrin reactions for examining the amino acid decomposing activity of Enterobacteriaceae.

Materials and methods

Organisms. Bacterial strains and their maintenance have been described [17—18].

Preliminary experiments. In order to find the most suitable routine technique, at first the dependence of alkalization and ninhydrin tests on various conditions was investigated. The following solutions were used: NH_4Cl, 0.1 g; $KH_2PO_4 \cdot 0.1$ g; $MgSO_4 \cdot 7H_2O$, 0.05 g; glycine, 0.01—0.20 g; nicotinamide, 0.001 g; distilled water, 100 ml; 1 per cent $CaCl_2$ solution, 0.01 ml; 1 per cent ferric ammonium citrate solution, 0.005 ml; indicator, 0.024—0.06 g; pH 4.3—8.3.

The result of the *alkalization test* (AT) was considerably influenced by the following factors: age of subculture (cultures stored for more than 24 hours showed a decreased activity), size of inoculum (mass seeding was advantageous), amount of substrate (optimum concentration was 0.2 per cent), volume of amino acid solution (at increased volumes the reaction velocity decreased), size of aerated surface (the activity increased at larger surfaces), initial pH of the amino acid solution (at lower pH values the activity decreased selectively), indicator and its concentration, presence of inhibiting substances (glycine was not broken down in solutions containing 0.01 per cent ethyl-mercuri-salicylate).

Ninhydrin test (NT). The ninhydrin reaction is used for the detection of amino acids [1, 4, 9, 15, 16]. Amino acids depleted of their amino group give no colour reaction when boiled with ninhydrin. The NT may be positive also with other compounds, such as ammonia, ammonium salts, iron compounds and substances containing alcohol, aldehyde or keto groups [7—10, 13]. In the present experiments the breakdown of amino acids was to be detected with the NT in media containing ammonium salts and iron compounds. Therefore a highly diluted (0.125 per cent) ninhydrin reagent was employed, which, when added in 0.2 ml portions to 2 ml amino acid-free medium, caused no coloration after 10 minutes' boiling.

Under the given experimental conditions the total amount of glycine was not always decomposed; partial breakdown could, however, also be shown with NT. In case of suitable enzyme activity no hydrindantin appeared in the examined tube when it was boiled for the time needed for the colour reaction to develop in the control (inhibitor-containing) solution.

Similarly to the result of AT, the result of NT was influenced by certain factors (age of subculture, volume of medium, size of free surface, pH). Other factors such as concentration of

substrate and indicator exerted an opposite effect. For the NT the application of dilute solutions was advantageous, since, as compared to the use of more concentrated solutions, in this manner the time of boiling was lengthened more considerably after 50 per cent of the amino acid had been broken down. The NT was more reliable in indicator-free solution.

Routine method. On the basis of preliminary experiments the following method has been adopted for the routine examination of glycine breakdown.

Preparation of glycine solutions. To 1 litre distilled water the reagents are added in the order: NH_4Cl, 1 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; nicotinamide, 0.01 g; 1 per cent $CaCl_2$, 0.10 ml; 1 per cent ferric ammonium citrate, 0.05 ml. After sterilization at 115°C for 15 minutes the stock solution can be stored at room temperature for months. For preparing AT medium, to a 100 ml portion of the stock solution 0.20 g glycine and 2.5 ml freshly prepared phenol red indicator (phenolsulphonphthalein, 0.01 g; N NaOH, 0.40 ml; distilled water, 4.6 ml) are added. The pH is adjusted to 5.6. For the preparation of NT medium, to the stock solution 0.05 per cent glycine is added without indicator, and the pH is adjusted to 7.2—7.4. The solution is then divided into two equal parts; to one of these 1 per cent ethyl-mercuri-salicylate is added after sterilization (control medium).

After sterilization at 115°C for 15 minutes the pH is checked and readjusted if necessary. The final medium can be stored for 3 weeks in the refrigerator.

The activity of several hundred strains was examined in pH 4.6, 0.05 per cent glycine solution prepared with phenol red indicator and in pH 6.5, 0.05 per cent glycine containing cresol red indicator. Both media gave good results.

Incubation and reading of AT. On the day of the examination the medium is distributed for the macrotest at 2 ml, for the microtest at 0.5 ml amounts into clean 16×160 mm Jena or Unihost test tubes. The tubes, cleaned previously from traces of acid or alkali, are stoppered with artificial cotton and sterilized. An ample amount (2 loops 2 mm in diameter) of a one day horse flesh infusion agar culture of the examined strain is suspended in the glycine medium. For the macrotest the tube is inoculated in a 37°C thermostat. For the microtest the tube is placed in a 40—41°C water bath for 4 hours then incubated in a 37°C thermostat overnight. Colour reaction in the macrotest tube is read daily, in the microtest tube after 4 hours and on the next day. We attempted to interpret the results according to the internationally used scheme [11]. The designations are shown in the key under Table I.

Technique and reading of NT. Inoculation of the indicator-free (pH 7.2—7.4) glycine and control medium with a one day culture is performed as described for AT. After 7 days' incubation 0.20 ml of 0.125 per cent aqueous ninhydrin is added to both tubes. The reagent should be freshly prepared or stored for not more than 3 days in the refrigerator. The tubes are placed in boiling water for about four minutes until the control medium shows a distinct blue colour, then cooled in water and read.

For the sake of parallelism with AT results, the reading of NT is considered positive or negative according to the presence or absence of enzyme activity. Thus, NT is positive (+) when the test suspension is colourless and the control assumes an intensive blue colour. Rarely occurring weak positive (±) reactions are indicated by a definite, but not intensive blue colour in the test suspension. When the test and control suspensions are similar, or the test suspension is darker in colour, the result is negative (—).

Both AT and NT give well reproducible results when the technical procedures are performed precisely.

Results

AT was carried out both in macro and micro forms; the micromethod was unsuitable for NT.

The result of AT is summarized in Table I. As the evaluation is arbitrary, it is obvious that there were differences in the data obtained with the two methods. As regards the most important differentiating criterium, however, the agreement was complete: neither method gave early alkalization with representatives of certain genera (species). The examined organisms were divided into 3 groups:

(1) *S. paratyphi* A, Shigella and Morganella gave no early positive reactions;

Table I

Result of alkalization test in glycine medium

	No. of strains	Macrotest					Microtest				
		–	(+)	+	(++)	++	–	(+)	+	(++)	++
E. coli	55	6	12	37	14	–	2	36	17	19	–
S. paratyphi A	7	6	1	–	–	–	6	1	–	–	–
Other Salmonella (*S. paratyphi* B, *typhi murium*, *bareilly*, *typhi*)	90	–	18	72	80	2	1	37	52	72	–
Klebsiella	25	1	2	22	21	1	4	6	15	14	–
Citrobacter	15	–	–	15	13	–	–	–	15	15	–
Enterobacter	15	1	–	14	8	4	1	3	11	12	–
Serratia	10	–	1	9	7	1	–	1	9	4	1
Arizona	15	–	4	11	14	1	–	1	14	13	1
Sh. dysenteriae	15	12	3	–	–	–	12	3	–	–	–
Sh. flexn. 3, 4, 5	45	39	6	–	–	–	41	4	–	–	–
Sh. boydii 1—15	30	27	3	–	–	–	22	8	–	–	–
Sh. sonnei	15	11	4	–	–	–	10	5	–	–	–
P. vulgaris	15	1	1	13	11	–	–	11	4	8	–
P. mirabilis	15	2	10	3	1	–	–	5	10	12	1
Morganella	15	12	3	–	–	–	14	1	–	–	–
Rettgerella	15	8	2	5	2	–	11	4	1	–	–
Providencia	15	7	4	4	2	1	11	2	1	–	–
Total	412	133	74	205	173	10	135	128	149	169	3

Key: Macrotest: – = No red colour 7 days; (+) = Red colour 4—7 days; + = Red colour 3 days; (++) = Intensive red colour 4—7 days; ++ = Intensive red colour 3 days.
Microtest: – = No red colour 24 hours; (+) = Red colour 5—24 hours; + = Red colour 4 hours; (++) = Intensive red colour 5—24 hours; ++ = Intensive red colour 4 hours.

(2) the majority of *E. coli*, *S. paratyphi B*, *S. typhi murium*, *S. typhi*, Klebsiella, Citrobacter, Enterobacter, Serratia, Arizona and *P. vulgaris* strains caused an early alkalization (mainly when tested with the macromethod);

(3) *P. mirabilis*, Rettgerella and Providencia strains were not uniform (most strains were negative, but some early or late reactions occurred).

Accordingly, the glycine decomposition test can be used for differentiation, as only those bacteria should be considered *S. paratyphi A*, Shigella or Morganella, which give no early positive AT. These organisms cause no intensive alkalization on further incubation either with the macro-or with the micromethod.

Results of NT are summarized in Table II. It is seen that glycine negative (*S. paratyphi A* and Shigella) and glycine positive (other Enterobacteriaceae) groups can be distinguished.

Table II

Result of ninhydrin test in glycine medium

	No. of strains	−	±	+		No. of strains	−	±	+
E. coli									
026	7	—	—	7	Klebsiella	30	—	—	30
055	8	1	—	7	Citrobacter	27	2	—	25
0111	8	1	—	7	Enterobacter	17	—	—	17
0124	29	—	—	29	Serratia	10	—	—	10
0126	6	—	—	6	Arizona	19	—	—	19
type strains	31	2	—	29					
Salmonella					*Shigella*				
paratyphi A	7	2	5	—	*dysent. 1—10*	18	18	—	—
paratyphi B	37	—	—	37	*flexn. 1*	20	20	—	—
stanley	6	—	—	6	*flexn. 2*	20	20	—	—
derby	6	—	—	6	*flexn. 3*	20	20	—	—
typhi murium	38	—	—	38	*flexn. 4*	20	18	—	2
kunzendorf	4	2	—	2	*flexn. 5*	8	8	—	—
bareilly	17	—	—	17	*flexn. 6*	19	19	—	—
bovis. morb.	3	—	—	3	*boydii 1—15*	20	20	—	—
typhi	40	—	—	40	*sonnei*	25	25	—	—
P. vulgaris	21	—	—	21					
P. mirabilis	23	1	—	22					
Morganella	15	—	—	15					
Rettgerella	19	—	—	19					
Providencia	15	—	—	15					
Total	340	9	5	326		273	170	—	103

It should be noted that in orientation experiments the method has proved suitable for the examination of further micro-organisms.

Discussion

In the available literature only a few data have been found as to glycine breakdown by bacteria. According to GALE [6] *E. coli* splits that substance by oxidative deamination into glyoxylic acid and ammonia; on the other hand, *Cl. sporogenes* produces acetic acid by anaerobic deamination. In STICKLAND's oxido-reduction reaction glycine is an H acceptor, while alanine may act as

an H donor; thus the two amino acids are decomposed through pyruvic acid into acetic acid, ammonia and carbon dioxide. EKLADIUS *et al.* [5], using a manometric method supplemented with paper chromatography for the examination of decarboxylase activity in *P. vulgaris*, found that in nitrogen atmosphere in the presence of 10^{-4} *M* pyridoxal, no appreciable amount of carbon dioxide is produced from glycine. In Warburg experiments STOKES and BAYNE [19] found that intensively multiplying salmonellae were oxidating glycine slowly; with less actively growing strains the reaction was also slow or absent. Glycine was oxidated through formate. ERLANDSON and RUHL [5*a*], examining the decomposition of 30 amino acids by the Warburg method, observed that *Sh. flexneri* 3 produced small amounts of ammonia from glycine.

Some attempts have been made to use glycine breakdown as a differentiation test. MEYER and CAMERON [12] performed manometric investigations into the action of Brucella, Moraxella, Neisseria and Bordetella strains on amino acids including glycine. They showed that the amino acid decomposing capacity of various species within one genus may be different. In Warburg experiments carried out by BAYNE and STOKES the examined 38 Salmonella strains decomposed glycine slowly; some *S. paratyphi A*, *S. abortus ovis*, *S. typhi suis* and *S. sendai* strains exerted no activity [2].

In RUHEMANN's ninhydrin test [15, 16] alpha-amino acids (including those incorporated in polypeptides and proteins), when heated with tricetohydrindenhydrate, are oxidated by giving off carbon dioxide and water into aldehyde and through some intermediary products the reagent is transformed into blue or red hydrindantin (diketohydrindyliden-diketohydrindiamine) [10]. ABDERHALDEN [1] applied NT for the diagnosis of pregnancy and cancer. His method, based on the detection of dialyzable substances produced from proteins disintegrated by enzymes (Abwehrfermente), however, failed to ensure the expexted results [14]. Recently ninhydrin has become an important reagent in paper chromatography for the detection of amino acids in protein hydrolysates [3, 4].

In the available literature no routine method has been found for the examination of microbial glycine decomposition and for the use of this reaction in the differentiation of bacteria. The simple technique of the present method allows the introduction of the reaction in every routine laboratory. Under conditions more or less differing from the optimal technique (alteration of pH or of the amount of the substrate) certain enteric bacteria may give reactions different from those obtained with other group or species. This observation may be useful in the characterization of such organisms. Glycine-negative shigellae and *S. paratyphi A* can well be differentiated from glycine-positive organisms. The best results for differentiation purposes are obtained with NT; the microtest gives the smallest number of positive reactions. As the latter is a rapid test, it can suitably be used in routine work.

Results given by AT and NT do not agree in all cases. For NT the bacteria are incubated in a solution approximately of the optimum pH value. The two tests differ in mechanism. Positive AT indicates the presence of alkali sufficient for a shift of the indicator; NT, on the other hand, is positive only when the glycine content decreases by approximately 50 per cent.

The present experiments have shown that the obtained results are valid for the described conditions only. A negative result does not yet indicate that the organism produces no glycine-decomposing enzyme. Under conditions more favourable for enzyme activity the same culture may decompose this amino acid.

It may be supposed that under the conditions described in the present paper glycine is oxidatively deaminated into glyoxylic acid and ammonia. This consideration is supported by the following findings. The process takes place only in the presence of oxygen (free air); when covered with liquid paraffin, otherwise positive cultures give negative alkalization reactions. Alkalization is promoted when the same volume of suspension is placed in a wider test tube, or when without changing the size of the test tubes smaller amounts of medium are used (in both cases the aerated surface of the suspension increases).

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SEROLOGICAL EVIDENCE OF THE INCIDENCE OF INFLUENZA EQUINE A-1 VIRUS INFECTIONS AMONG HORSES IN HUNGARY

By

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Summary. Horses in 12 stocks were screened for haemagglutination-inhibiting antibodies to the subtypes equine A-1 and A-2, human A-2 and swine A-1 of influenza virus. Eight-hundred and thirty-five sera, 487 of which originated from race-horses, were tested. Antibodies were found only to subtype equine A-1. The incidence of these antibodies was high in horses over 3 years of age in each of the tested stocks. Colts 2 and 3 years of age were tested in 4 stocks; of these colts 49 per cent, on the average, had equine A-1 antibodies in two closely connected stocks. In the other two the colts were practically seronegative. It is concluded that equine A-1 virus was highly disseminated in Hungary before 1961 and has persisted in certain horse stocks.

In the last 10 years explosion-like outbreaks of an acute disease limited to the upper respiratory tract, similar to human influenza, have been observed in horses, first in Europe, later also in the Americas.

The first outbreak was observed in Sweden in 1955, by Heller *et al.* [1]. Similar outbreaks were reported not much later from Czechoslovakia, where Sovinova *et al.* [2] succeeded in isolating a virus strain in 1956. The strain named influenza equi A (Prague) 56 proved to represent a new subtype of influenza A virus. Subsequently, similar outbreaks showing serological evidence of infection with the same virus were reported from other European countries as well [3—8].

In the U. S. A. serological screening of horses resulted in demonstrating antibodies to the same virus. It was shown that the virus had been present in the U. S. A. at least from 1957.

In the Miami area, during an explosive respiratory outbreak among race-horses an influenza virus strain different from strain equi A (Prague) 56 was isolated from the nasal discharge of a febrile horse [10, 11]. The subtype represented by the new strain has been termed influenza equine A-2 to differentiate it from the subtype represented by the Czechoslovak strain, which then was termed influenza equine A-1.

At present these two equine subtypes of influenza A virus are known.

In the U. S. A. during the epizootic caused by the equine A-2 virus, equine A-1 strains were also isolated [12]. A great number of the horses that had been infected by the latter were subsequently infected by equine A-2.

Early in 1965 a strain classifiable into the equine A-2 subtype was isolated in England from horses with acute respiratory illness [13]. Somewhat earlier complement-fixing antibodies to the equine A-2 virus were demonstrated in horse sera in Switzerland [14].

The aim of the present investigation carried out in cooperation with the Division of Veterinary Public Health of the WHO, was to study the occurrence in Hungary of the two equine subtypes of influenza A virus.

Materials and methods

Horses. Eighthundred and thirty-five horses were tested. The horses belonged to 12 different stocks, of which 5 (stocks A, G, S, T and U) consisted of race horses; the colts in farm K were reared to be draught horses; the remaining horses, all draught horses over three years of age, were kept in six large state farms.

The birth year of the race horses and of the colts in stock K was registered; that of the draught horses was unknown.

As most of the horses were born in the first quarter of the year, the age given in the text and in the Tables means just completed years for some horses; others were younger by one or two months than given.

Sera. Serum specimens were inactivated by heating at 56°C for 30 minutes and stored in the frozen state. Before tested, the sera were treated with KIO_4 at 37°C.

Influenza virus strains. The strains equine A-1 Czech horse flu/59, human A-2 Singapore 1/57 and swine A-1 Shope 15 were kindly supplied by the World Influenza Centre (London), the strain equine A-2 Miami/63 by Dr. FLORENCE LIEF (Philadelphia).

Haemagglutination inhibition (HI) tests. The HI test was performed in TAKÁTSY's Microtitrator apparatus [15], using 0.025 ml loops, twofold dilution series of serum, and chicken erythrocytes. Eight haemagglutinating units of virus were added to each serum dilution. The titre value means the serum dilution, as calculated for the volume of the serum-virus mixture, exhibiting partial inhibition. For the cases when a dilution causing complete inhibition was followed by complete haemagglutination in the presence of the next serum dilution, interpolated titre values (1 : 6, 1 : 12, *etc.*) are given.

Results

HI antibodies to subtypes equine A-2, human A-2 and swine A-1 attained the level 1 : 8 in none of the 835 equine sera tested. In contrast, the HI antibodies to the equine A-1 subtype attained or even exceeded this level in 234 of the 487 sera originating from race horses (48 per cent) and in 63 to 76 per cent of the horses kept in large state farms. In the following the data of the 487 race horses will be analyzed.

Distribution of the sera by age of the donor horse and the HI titre of the serum is shown in Table I. The data for horses from 6 to 10 years of age have been combined as there was no appreciable difference in titre distribution between the 6- and 7-year-old horses and the 8—10-year-old ones. For similar reasons the values for horses 11 to 20 years of age have also been combined.

Table I shows that the frequency of titres 1 : 4 and 1 : 6 was considerably lower than that of each of the titre values from 1 : 8 to 1 : 48, *i.e.* the titre distribution curve had two peaks (Fig. 1). Taking into account that titres up

Table I

Distribution of 505 horses by age and antibody titre to influenza equine A-1 virus

HI titre*	Age in year							
	1	2	3	4	5	6—10	11—20	Total
64—96	—	—	1	1	2	3	3	10
32—48	—	—	3	2	8	25	8	46
16—24	—	4	10	4	12	41	16	87
8—12	2	4	14	20	9	34	8	91
4— 6	3	7	7	3	2	12	4	38
Negative	63	59	43	26	13	24	5	233
Total	68	74	78	56	46	139	44	505

* Reciprocals

to 1 : 6 occurred also in relation to the other three subtypes, the titres $<1:8$ should not be accepted as indicative of a previous infection with the homologous subtype. On the other hand, the separation of horses yielding titre $\geqslant 1:8$

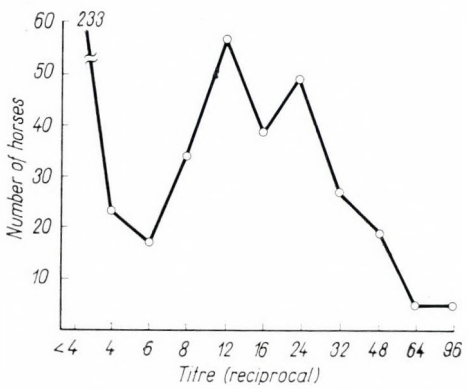

Fig. 1. Distribution of 505 horses by HI titre to influenza equine A-1 virus

from the negative horses by a minimum supports the view that titres higher than 1 : 6 may be accepted as specific.

Fig. 2 shows that the percentage of seropositive horses tended to rise with age. Yet, some 2- and 3-year-old horses and even two colts one year of age had a HI titre to the subtype equine A-1.

Table II shows the incidence of HI antibodies to subtype equine A-1 in each of the five stocks of race-horses. It is clearly seen that from 4 years of age upwards these antibodies were equally frequent in each stock. Two- and three-year-old horses were tested only in four stocks. A considerable number of

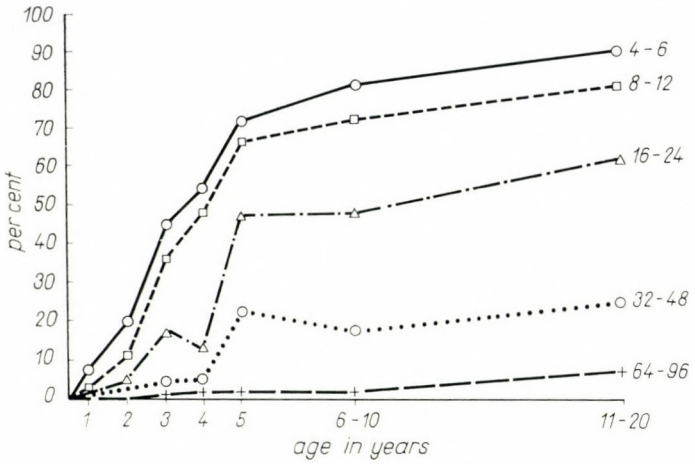

Fig. 2. Percentage incidence of anti-equine A-1 HI antibodies attaining or exceeding the titres indicated in the figure

Table II

Distribution of 505 horses in six stocks by age and serological status

Stock	Age in year							Total
	1	2	3	4	5	6—10	11—20	
A	—	0/26	1/17	6/15	2/6	4/6	—	13/70
G	—	0/25	1/15	6/13	1/4	5/5	—	13/62
S	2/50	5/14	20/30	7/13	6/9	42/62	28/37	110/215
U	—	3/9	6/16	6/10	14/15	11/14	—	40/64
T	—	—	—	2/5	8/12	41/52	7/7	58/76
K	0/18	—	—	—	—	—	—	0/18
Total	2/68	8/74	28/78	27/56	31/46	103/139	35/44	234/5ß5
Percentage for A+G		2			49			
S+U		49			70			

Numerator: number of cases with titre 1 : 8 to influenza equine A-1.
Denominator: total number of cases

seropositive horses of this age group were found in two stocks (S and U). In the other two (stocks A and G) none of the two-year-old horses had antibodies. Of the three-year-old horses two were seropositive (one in stock A — and one in stock G, with titres 1 : 48 and 1 : 12, respectively).

One-year-old colts were tested in two stocks (S and K). The two seropositive colts (titre, 1 : 12 in both cases) belonged to stock S, where a considerable number of the two- and three-year-old horses were also positive. From stock K serum samples taken from 18 farm colts were tested. All of these proved to be negative.

Discussion

The present data show that in the race-horses tested the equine A-2, human A-2 and swine A-1 subtypes of influenza virus were absent, whereas more than two-thirds of both the race-horses and farm-horses over 3 years of age had been infected with the equine A-1 subtype. This is not surprising, being consistent with data published from some other European countries.

Taking into account that most recently the equine A-1 subtype can rarely be demonstrated, occurrence of seropositive animals was less expectable among two- and three-year-old horses. However, all the seropositive colts but two, belonged to two closely connected stocks. It seems that during the recent few years the occurrence of equine A-1 virus has not been general among horses in Hungary. Nevertheless, the virus has persisted in several stocks.

The presence of seronegative horses, independent of age, in each of the stocks tested might be explained in two ways.

(i) A considerable proportion of the horses escaped infection when the virus was circulating.

(ii) Considerably more horses had been infected than those having detectable antibodies, but since then the antibodies have disappeared from the sera of many horses.

The fact that the titre-distribution curve has two peaks speaks against the second explanation and suggests that the distribution of the horses was heterogeneous in each of the stocks tested. This might be explained by the supposition detailed under (i) or, else, one might assume that most of the horses showing titres acceptable as specific had been infected twice, and only the reinfected horses have persistent titres; presumably, after a single infection the serum titre soon falls below the detectable level. This assumption would be consistent with the experience in humans. The possibility of reinfection would also explain the persistence of the virus in a sufficiently large stock of horses over years in which only sporadic infections occur.

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PROPOSALS AND RECOMMENDATIONS OF THE PROVISIONAL COMMITTEE FOR NOMENCLATURE OF VIRUSES (P. C. N. V.)

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Foreword

It has been felt for a long time that an international agreement on nomenclature of viruses is a necessity. In theory "the international code of nomenclature of bacteria and viruses" should have been applied to viruses. The Judicial Commission of the International Committee of Bacterial Nomenclature therefore nominated a subcommittee of viral nomenclature (S. C. V. N.). The S. C. V. N. wisely decided that "The Bacteriological Code" could not be applied to viruses. However no step was taken by the S. C. V. N. in order to promote a specific "viral code". As a matter of fact the S. C. V. N. had no legal power to do so.

Nevertheless it was obviously necessary and urgent that viruses should not be left in a taxonomic vacuum. The Executive Committee of I.A.M.S. discussed the matter with the chairman of the S. C. V. N. *Sir* CHRISTOPHER ANDREWES who in turn consulted the members of this Committee, and with their approval, dissolved the subcommittee.

After consultation with the Advisory Council of I.A.M. S. with the Chairman of the Judicial Commission of the International Committee of Bacterial Nomenclature and with the exchairman of the dissolved S. C. V. N., the Executive Committee of I. A. M. S. (E. C. I.A.M.S.) took following action at its Paris Meeting in July 1963. E. C. I.A.M.S.:

(a) took notice of the willingness of most of the members of the subcommittee on viruses to dissolve this subcommittee,

(b) decided that a new international committee, the International Committee on Nomenclature of Viruses (I. C. N. V.) be created, the counterpart of the International Committee on Bacteriological Nomenclature (I. C. B. N.)

(c) decided that the members of the I. C. N. V. (like those of the I. C. B. N.) would be nominated by the National Societies. The first official meeting would be held during the IXth Congress in 1966 in Moscow.

Moreover, E. C. I.A.M.S. decided to create a provisional Committee, the P. C. N. V., whose task was to prepare the work of the future I. C. N. V. and of its Judicial Commission. E. C. I.A.M.S. nominated the members of the P. C. N. V. after consultation with the President of the I. C. B. N., the

chairman of the S. C. N. V. and the president of the Advisory Council of I. A. M. S.

The members of the P. C. N. V. are: C. H. ANDREWES (Coombe Bissett, Salisbury), P. D. COOPER (Canberra), S. FAZEKAS DE ST GROTH (Canberra), H. S. GINSBERG (Philadelphia), D. GOLDFARB (Moscow), L. HIRTH (Strasbourg), G. IVÁNOVICS (Szeged), M. KAPLAN (Geneve), A. LWOFF (Paris), K. MARAMAROSCH (Yonkers, New-York), H. PEREIRA (Mill Hill, London), P. TOURNIER (Paris) and V. ZHDANOV (Moscow).

The Committee elected a President, *Sir* CHRISTOPHER ANDREWES (Overchalke, Coombe Bissett, Salisbury, Wiltshire) and a Secretary, PAUL TOURNIER [Centre de Recherches sur le Cancer, B. P. No 8, Villejuif (Seine)].

The Committee discussed the problem extensively by correspondence and held its first — and last — meeting in Paris on June 25 and 26, 1965. (Dr. FAZEKAS DE ST GROTH and Dr. GOLDFARB were unable to attend the meeting).

The main task of the P. C. N. V. is, as already said, to prepare the work of the future Judicial Commission. It should be clear that only the Judicial Commission of the I. C. N. V. will have the legal power to enforce a code of nomenclature and to take decisions concerning nomenclature of viruses. The P. C. N. V. has no right to take decisions, and can only make proposals or recommendations.

The members of the P. C. N. V. belong to widely different fields of virology and many of them had diverging or even completely opposite views on the problem of viral nomenclature. In order that an agreement be reached, everyone had to sacrifice some of his own ideas or feelings. As a result of a remarkable cooperative spirit all the "decisions" were unanimous or quasi-unanimous.

Proposals

Principles. (1) An International Nomenclature of Viruses is necessary. Viruses cannot be left in a taxonomic vacuum.

(2) The only way to achieve an International Nomenclature of viruses is by a binominal system.

(3) The code of nomenclature of bacteria cannot be applied to viruses.

(4) Virologists will therefore have to build their own code.

Comments. The bacteriological code, as well as the Zoological and Botanical codes, includes the law of priority. To apply the law of priority to viruses would be almost impossible and would certainly be the cause of endless polemics. The decision as to which names should be maintained will be in the hands of the Judicial Commission (J. C.) of the International Committee of the Nomenclature of Viruses (I. C. N. V.).

Naming of taxa. The names already in use should be maintained whenever it is possible. For the formation of new names of taxa, the P. C. N. V. recommends:

(1) No taxon should be named from a person.

(2) Anagrams, siglas, hybrids of names, non-sense names should be prohibited.

(3) Names should preferentially be latin or latinized greek names (see Zoological, Botanical and Bacteriological codes).

(4) A species shall be selected as being the most fully defined by published work (*e.g.* by serotype and other criteria) and should desirably be represented in a Type Culture Collection and held and maintained in replicate in 4 or 5 different countries. The task of describing genera and type species and of making detailed proposals for classification shall be left to individual authors or subcommittees of the future Judicial Commission.

(5) A subgeneric name is formed by an epithet in brackets following the name of the genus. Example: *genus Cocksackievirus, subgenus: Cocksackievirus (A).*

(6) Different species belonging to different *subgenera* of the same *genus* can have the same name. Example: *Cocksackievirus (A) primus, Cocksackievirus (B) primus.*

Comments. This rule was adopted in order to be able to keep systems already in use. It implies that whenever a *genus* be subdivided into *subgenera*, the name of the *subgenera* must be mentioned.

(7) Specific names can be names, as in Zoology, Botany or Bacteriology but can also be letters or numerals. Yet the type species must have a latinized name. Example: Cocksackievirus (A) primus would be the type species. The other species could be 5, 18, α, β, etc. . . . This again will make it possible to maintain as valid, systems already in existence.

(8) The names of all viral genera end in "virus", example: *Poliovirus, Napovirus.*

(9) No viral genus names should be identical with the genus of a bacterium, a protozoon, an alga, that is, with the names of procaryotic or eucaryotic protists. The genera of viruses having to end with the suffix virus, this rule is therefore necessary only if, for example, the name of protist ends with the same suffix virus. If a bacterium is called Chlamydovirus, no viral genus can bear this name.

(10) A genus shall be selected to typify each family.

(11) A family is named from its type genus. Example: *Poxvirus, Poxviridae.*

(12) The suffix for the family is *idae*, as in the zoological code. This ending was preferred to *aceae*, in use for plants and bacteria because: (a) it is more euphonious; (b) it allows discrimination between viral and bacterial families.

(13) All viral genera ending in *virus* and all families being named from their type genus, it follows that all the viral families end with *viridae.*

Table 1

(A)

Family (-viridae)	*Genus* (-virus)	*Type species*	*Common name*
Poxviridae	1. *Poxvirus* (type genus)	*variolae*	Variola
	2. *Dermovirus*	*orfi*	Contagious pustular dermatitis
	3. *Pustulovirus*	*ovis*	Sheep pox
	4. *Avipoxvirus*	*galli*	Fowl pox
	5. *Fibromavirus*	*myxomatosis*	Rabbit myxoma
	6. *Molluscovirus*	*hominis*	Molluscum contagiosum
Microviridae	1. *Microvirus* (type genus)	*monocatena*	Phage φX 174
Parvoviridae	1. *Parvovirus* (type genus)	*ratti*	Kilham Rat Virus
Papillomaviridae	1. *Papillomavirus* (type genus)	*sylvilagi*	Shope papilloma
	2. *Polyomavirus*	*neoformans*	Polyoma virus
Adenoviridae	1. *Adenovirus* (type genus)	(*hominis*) *quintus*	Adenovirus 5
Iridoviridae	1. *Iridovirus* (type genus)	*tipulae*	Tipula iridescens
Inophagoviridae	1. *Inophagovirus*	*bacterii*	fd phage (Hoffmann Berling)
Herpesviridae	1. *Herpesvirus* (type genus)	*hominis*	Herpes simplex virus
Phagoviridae	1. *Phagovirus* (type genus)	(*coli*)*T secundus*	Phage T 2
Dolichoviridae	*Dolichovirus* (type genus)	*brassicae*	Cabbage mosaic
Protoviridae	*Protovirus* (type genus)	*tabaci*	Tobacco mosaic
Pachyviridae	*Pachyvirus* (type genus)	*crotalum*	Rattle mosaic
Leptoviridae	*Leptovirus* (type genus)	*solanum*	Potato X
Mesoviridae	*Mesovirus* (type genus)	*pisum*	Pea mosaic
Adroviridae	*Adrovirus* (type genus)	*trifolii*	White clover mosaic
Myxoviridae	1. *Myxovirus* (type genus)	(*influenzae*) *A*	Influenzae A
	2. *Rabiesvirus*	*canis*	Rabies
	3. *Sigmavirus*	*drosophilae*	Virus of L'Héritier
Paramyxoviridae	1. *Paramyxovirus* (type genus)	(*para influenzae*) *primus*	Myxovirus parainfluenzae 1
	2. *Bronchovirus*	*syncytialis*	Respiratory syncytial
Stomatoviridae	*Stomatovirus* (type genus)	*bovis*	Vesicular stomatitis

(B)

Family (-viridae)	*Subfamilies* (-virinae)	*Genus*	*Type species*	*Common name*
Napoviridae	*A. Napovirinae* (nominative subfamily)	*Napovirus* (type genus)	*flavicans*	Turnip yellow mosaic
	B. Picornavirinae	*1. Picornavirus* (type genus)	*aphtae*	Foot and mouth disease
		2. Poliovirus	*primus*	Polio 1
		3. Coxsackievirus	(*A*) *primus*	Coxsackie A1
		4. Echovirus	(*hominis*) *primus*	Echo 1
		5. Rhinovirus	(*hominis*) *primus*	Rhinovirus 1
		6 Cardiovirus	*ratti*	EMC
	C. Androphagovirinae	*Androphagovirus* (type genus)	*bacterii*	RNA phage

(C)

Family (-viridae)	*Genus*	*Type species*	*Common name*
Reoviridae	*1. Reovirus* (type genus)	(*mammalis*) *primus*	Reovirus 1
	2. Neovirus	*neoformans*	Wound tumor virus
Arboviridae	*Arbovirus* (type genus)	*occidentalis*	WEE

(14) It is proposed that the terms, phylum, division, class, order, family, genus and species are applied to viral taxa. This does not imply any homology between viral taxa and taxa belonging to other phyla.

(15) Depersonalisation. The name of a taxon, whatever its rank, is not followed by the name of the author who proposed it. Example: If SMITH-DUPONT proposed the genus *Poxvirus*, the genus is *Poxvirus* and not *Poxvirus Smith-Dupont*.

(16) In order to be valid a name has to be endorsed by the Judicial Commission. However the Judicial Commission can only decide that a name is correctly formed, that is in agreement with the rules, but has no right to reject a name on the ground, for example, that it is unnecessary. In other terms, the Judicial Commission deals with nomenclature and not with systematics. The necessity of a name, its justification can be studied by a specialized Committee or by an individual. It is clear that unnecessary names will on the long run die out.

Nomina conservanda. The P. C. N. V. considered that it would be useful if virologists could already agree on names which should be maintained. The P. C. N. V. therefore propose a list of *nomina conservanda* and a list of new genera. A type species was selected for each genus.

Classification of viruses

As stated already the P. C. N. V. has to deal with nomenclature and noth with systematics. As for any virologist, the P. C. N. V. has, however, the right to propose or to adopt a system. Moreover it has also the right to propose names for the various taxa. Taking advantage of these rights, the P. C. N. V. has discussed the principles of classification and reached the following conclusions: (a) a system of viruses should embrace the viral world as a whole; (b) the criteria for the definition of divisions, classes, orders and families should be clearly defined.

The L. H. T. (LWOFF, HORN, TOURNIER) system was found to be the most suitable for the time being and is recommended as a provisional system.

Principles. (a) The virion shall be the basis of the taxonomy. (b) The following 4 characters of the virion shall be used for the definition of families without specifying any hierarchical significance at this stage.

(I) The chemical nature of the nucleic acid *(i.e.* RNA or DNA)

(II) The symmetry of the nucleocapsid *(i.e.* helical, cubical or binary)

(III) The presence or absence of an envelope for which the name *peplos* is proposed. A peplos is formed of *peplomers* just as a capsid is formed of capsomers

(IV) For helical viruses: the diameter of the nucleocapsid. For cubical viruses: the number of triangulation and the number of capsomers.

The sum of these four criteria will define the family.

Classification. The hyerarchy and taxa proposed by L. H. T. were discussed. The first criterion, which was proposed by P. D. COOPER, is the nature of the nucleic acid hence two *divisions: deoxyvira* and *ribovira.* The second criterion is the type of symmetry, whether cubical or helical. Hence four *classes: deoxyhelica, deoxycubica, ribohelica, ribocubica.* A group of viruses, bacteriophages possessing a tail, exhibit a double type of symmetry, that of the head and of the tail, that is a binal symmetry. As they are deoxyvira, a fifth class is proposed deoxybinala. If it is later found that some *ribovira* have a tail they would be called *ribobinala.* Then, each class is divided into orders according to whether the nucleocapsid is naked or enveloped.

The names of the orders will be found in Table II. All the viruses possessing an envelope or peplos have been given a name which includes a prefix corresponding to a greek or latin name meaning something like mantle.

The orders are subdivided into families according to the number of capsomers and the triangulation number (viruses with cubical symmetry) or according to the diameter of the nucleocapsid (viruses with helical symmetry). As the families are named from their type genus, no problem of nomenclature is posed by the naming of family.

An alteration of the L. H. T. system proposed by HIRTH was adopted by the P. C. N. V. The viruses possessing RNA, helical symmetry and a naked nucleocapsid, the order of *Rhabdovirales*, is subdivided into two suborders according to the rigidity or flexibility of the virion. Each suborder is then subdivided into families according to the diameter of the nucleocapsid. The reason for this procedure have been discussed by HIRTH.

The code

The P. C. N. V. decided that it will prepare a tentative code "Code of the nomenclature of viruses". The code will be circulated to the members of the I. C. N. V. as soon as possible.

During the IXth Congress, the Judicial Commission will have to study the tentative P. C. N. V. code, its amendments or other codes proposed by virologists. It is hoped that an agreement can be reached in Moscow and that a code will be available during the year 1966. The council for the International Organization of Medical Sciences has already agreed to study the problem of its publication.

It would help the P. C. N. V. and the future Judicial Commission if the proposals concerning the code were circulated as long in advance as possible.

In order to facilitate the work of the P. C. N. V., the National Societies are asked to send the name of their national nominates, not only to the Secretary General of I. A. M. S. but also to the Chairman and to the Secretary General of the P. C. N. V.

Additional remarks

(1) A few binominal systems of nomenclature have been proposed in the past. One of the reason for which they were not adopted is that they involved a radical change of universally accepted names. The P. C. N. V. has taken great care not to suppress names which are in common use.

(2) A number of names such as, for example, *Echo*, *Reo* are not in agreement with the rules proposed by the P. C. N. V.: they are sigla. Nevertheless the P. C. N. V. proposes them as *nomina conservanda* because they are in common use.

(3) *Picorna* is now commonly used in order to designate a "group" of viruses. In order to be able to maintain this name for a taxon above the rank

Table II

Phylum	Subphyla	Classes	Orders	Sub-orders	Families		
	Type of genetic material	Symmetry of the nucleocapsid H : helical C :cubical B : binal	Nucleocapsids Naked (N) or Enveloped (E)	Rigid (R) or Flexuous (F)	For helical viruses: diameter of the nucleocapsid For cubical viruses: number of triangulation* and number of capsomers		
Vira	DNA Deoxyvira	H Deoxyhelica	N —				—
			E Chitovirales		100 Å		Poxviridae
		C Deoxycubica	N Haplovirales			1— 12	Microviridae
						3— 32	Parvoviridae
						7— 72	Papilloviridae
						25—252	Adenoviridae
						81—812	Iridoviridae
						?	Inoviridae
			E Peplovirales			16—162	Herpesviridae
		B Deoxybinala	N Urovirales				Phagoviridae
			E —				—
	RNA Ribovira	H Ribohelica	N Rhabdovirales	R: Rigidovirales	120—130 Å		Dolichoviridae
					150 Å		Protoviridae
					200 Å		Pachyviridae
				F: Flexivirales	100—110 Å		Leptoviridae
					120—130 Å		Mesoviridae
					150 Å		Adroviridae
			E Sagovirales		90 Å		Myxoviridae
					180 Å		Paramyxoviridae
							Stomatoviridae
		C Ribocubica	N Gymnovirales			3— 32	Napoviridae
						9— 92	Reoviridae
			E Togavirales			?	Arboviridae

*See Annex 1

of genus and lower than an order, that is a family or a sub-family, it was necessary that a genus be named *Picorna*. This was done.

(4) *Papova*, which is a hybrid of *Papilloma*, *Polyoma* and *Vacuolating agent* and does not correspond to a genus, could not be maintained.

Annex I

Definition of the triangulation number. A polyhedron whose faces are all equilateral triangles is called a deltahedron. Deltahedra models can be constructed from-folded cardboard nets of equilateral triangles. We have enumerated all possible deltahedra which have icosahedral symmetry ("icosadeltahedra") [CASPAR & KLUG, 1962]. The icosahedron itself has 20 equilateral triangular faces, and any icosadeltahedron has 20 T facets, where T is the *triangulation number* given by the rule: $T = Pf^2$ where P can be any number of the series 1, 3, 7, 13, 19, 21, 31, 37 . . . ($= h^2 + hk + k^2$, for all pairs of integers h and k having no common factor) and f is any integer. For a fixed value of P, increases in f from 1 upward correspond to successive subtriangulations of the primitive deltahedron (Table III).

Table III

The classes of icosahedral deltahedra

Tabulation of the Triangulation Number T

(CASPAR, D. L. D., and KLUG, A.: Cold Spr. Harb. Symp. quant. Biol. 27, 15, 1962)

Class										
P = 1	1	4	9	16	25	.....				
P = 3	3	12	27	.....						
Skew classes	7	13	19	21	.....					

Triangulation N° T = Pf^2 where $P = h^2 + hk + k^2$, h and k any pair of integers with no common factor and f = 1, 2, 3, 4, ...

No. of structure units S = 60 T

No. of morphological units M = 10 T + 2
= 10 (T−1) hexamers + 12 pentamers

Some established virus examples

Phage ΦX, T = 1; Turnip yellow mosaic virus T = 3; Herpes, Varicella T = 16; Adenovirus Infectious canine hepatitis T = 25.

Annex II

Glossary—glossaire

Adros (gr.)	Thick	Epais
Andreios (gr.)	Male	Mâle
Aphtai (gr.)	Ulcerating vesicles in the mouth	Aphtes
Avis avis (lat.)	Bird	Oiseau
Beta bêtae (lat.)	Beet	Betterave
Bos bovis (lat.)	Cow	Vache
Brassica brassicae (lat.)	Cabbage	Chou
Cavia caviae (lat.)	Guinea pig	Cobaye

Chiton (gr.)	Tunic	Tunique
Cricetus criceti (lat.)	Hamster	Hamster
Crotalon (gr.)	Rattle	Grelot
Dolichos (gr.)	Long	Long
Equus equi (lat.)	Horse	Cheval
Flavus (lat.)	Yellow	Jaune
Flecto, ere (lat.)	To bend	Courber
Gallus galli (lat.)	Cock	Coq
Gymno (gr.)	Naked	Nu
Haploos (gr.)	Simple	Simple
Iris iridos (gr.)	Iris, Rainbow	Iris, arc-en-ciel
Is inos (gr.)	Fiber, filament	Fibre, filament
Leptos (gr.)	Narrow	Etroit
Mesos (gr.)	Middle	Moyen
Napus napus (lat.)	Turnip	Navet
Ornis ornithos (gr.)	Bird	Oiseau
Oura ouras (gr.)	Tail	Queue
Ovis ovis (lat.)	Sheep	Brebis
Pachus (gr.)	Thick	Epais
Parvus parvi (lat.)	Small	Petit
Peplos (gr.)	Woman mantle	Manteau de femme
Pisum pisi (lat.)	Pea	Pois
Protos (gr.)	First	Premier
Rana ranae (lat.)	Frog	Grenouille
Rhabdos, dou (gr.)	Rod	Baguette
Rigidus (lat.)	Rigid	Rigide
Sagum sagi (lat.)	Mantle	Saie
Simia simiae (lat.)	Monkey	Singe
Solanum solani (lat.)	Night shade	Solanée
Stoma stomatos (gr.)	Mouth	Bouche
Sus suis (lat.)	Pig	Porc
Sylvilagus (lat.) (Zoological name of the genus cottontail)	Cottontail (Wild rabbit)	Lapin sauvage du continent américain
Toga togae (lat.)	Toga (Roman mantle)	Toge
Trifolium ii (lat.)	Clover	Trèfle

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EPISOMIC ANTIBIOTIC RESISTANCE AMONG SHIGELLA STRAINS ISOLATED IN HUNGARY

By

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(Received May 4, 1965)

Summary. The episomic nature of antibiotic resistance could be verified for 102 out of 103 Shigella strains with different antibiograms, isolated in the period 1964—65.

Transfer of episomic resistance to a *Serratia marcescens* strain was successful with 61 per cent of 83 Shigella strains. Treatment with acridine orange of 62 strains gave positive results in 93 per cent. The success or failure of transfer had a demonstrable relationship with the antigenic structure.

The majority of R-factors exhibited segregation in both transfer and drug resistance elimination experiments. The trend and frequency of segregation differed in the elimination and transfer experiments and with the spontaneous loss of R-factor.

Theoretically, the incidence of bacterium strains resistant to a single antibiotic should exceed that of those resistant to two or more antibiotics. With numerous species, however, the overwhelming majority of strains proved to be multiple resistant. Basically this fact led Japanese authors to the discovery of the episome responsible for multiple drug resistance (R-factor).

In a previous paper one of us [1] reported on multiple resistance in about two thirds of the 1200 resistant Shigella strains isolated from 1961 to 1964. This observation initiated the study of the occurrence of episomic antibiotic resistance among the Shigella strains isolated in Hungary.

Materials and methods

Strains. A total of 103 resistant Shigella strains, isolated and submitted for identification in the period 1964—65, was selected for the investigations. After biochemical control, the strains were examined for antigenic structure; the *Sh. flexneri* strains were also typed, using factor sera.

Assay of antibiotic resistance. For the qualitative determination of resistance the paper disk method was used. With streptomycin (Sm), chloramphenicol (Cm) and tetracyclines (Tc), the antibiotic content of each disk was adjusted to 30 μg. Quantitative determination of resistance was performed with the dilution technique.

Transfer experiments. Transmission of the R-factors from Shigella strains was carried out with the conjugation technique. As recipient a sensitive strain of *Serratia marcescens* (No. 229) was used. To 5 ml of a 18-hour broth culture of the recipient 0.5 ml of a 18-hour broth culture of the donor (Shigella) strain was added and the mixture incubated for 2 hours at 37° C. Then 0.5 ml of the mixture was plated on Endo-agar containing 50 μg/ml of the examined antibiotic.

Contraselection of the donor strain was carried out by previous plating with 0.2 ml of virulent phage lysate. The phage strains F_1, F_2, F_8, F_9 and F_{12} were isolated and kindly supplied by Prof. ST. SŁOPEK (Wroclaw). The phage sensitivity of the Shigella strains was tested in preliminary experiments by the method of MULCZYK and LACHOWICZ [2].

Frequency of transfer was calculated per original donor cell.

Treatment with acridine dye. For this purpose acridine orange was used throughout. As a rule the strains were cultured for 18 hours in broth containing 50 μg/ml of acridine orange. Some strains were carried through ten serial passages in such medium. Serial dilutions of the culture were further examined by plating. From plates exhibiting an appropriate number of colonies (20 to 50), transfer were made by LEDERBERG's replica plating method [3] to plates containing 50 μg/ml of the appropriate antibiotic and to plain agar plates as well. Missing colonies of replicas grown on the antibiotic containing agar were estimated by comparison with plain replicas.

In some cases non-resistant colonies encountered on the plain agar were further examined by qualitative and quantitative antibiotic resistance tests.

Spontaneous loss of the R-factor. Resistant colonies of some representant strains were carried through 1 or 10 passages in broth to study the spontaneous loss of the R-factor.

Results

Test material. The antigenic structures and antibiograms of the examined strains are shown in Table I.

The antigenic structures observed were partly congruent with the relative frequency of types, namely of *Shigella flexneri* 2a, 3 and 1b. By aimed selection

Table I

Antigenic structure and antibiogram of Shigella strains examined for nature of antibiotic resistance

	Sm, Cm, Tc	Sm, Cm	Sm, Tc	Cm, Tc	Tc	Sm	Cm	Total (strains)
Sh. flexneri 1a	12	2	—	—	1	—	1	16
,, 2b	14	5	3	—	2	3	1	28
,, 2b	2	—	—	—	—	—	—	2
,, 3a	9	1	—	—	8	3	—	21
,, 4a	—	2	—	—	—	—	—	2
,, var. X	—	—	—	—	—	—	1	1
Sh. sonnei	10	8	1	3	8	—	2	32
Sh. boydii	1	—	—	—	—	—	—	1
Total	48	18	4	3	19	6	5	103

Sm = Streptomycin-resistant; Cm = chloramphenicol-resistant, Tc = tetracycline-resistant

of the strains only about 50 per cent of them were of the "classic" multiple resistant type (Sm, Cm, Tc), while the rest included other multiple resistant types — Sm, Cm — and strains resistant to a single antibiotic. Among these, in accordance with our test material, the majority were tetracycline (Tc) resistant.

2. *Transfer experiments.* Transmission of resistance to a sensitive strain of *Serratia marcescens* designated "229" was attempted with most Shigella strains, a total of 83. With the rest, contraselection was excluded by phage

resistance. Out of the 83 Shigella strains belonging to various serotypes, transfer was successful in 51 cases. Results of transfer experiments in relation to antigenic structure and antibiogram of the strains are shown in Table II.

It appears that the success of transfer has a relationship with the antigenic structure, as 23 successful transfers were registered among 24 *Shigella sonnei*

Table II

Transmissibility of R-factor in relation to antigenic structure and antibiogram

Antibiogram	Transfer	*Sh. flexneri*				*Sh. sonnei*	Total (strains)
		1b	2a	3	4a		
Sm, Cm, Tc	Positive	11	3	4	.	6	24
	Negative	1	9	3	.	0	13
Sm, Cm	Positive	1	1	0	2	6	10
	Negative	1	3	1	.	1	6
Sm, Tc	Positive	.	0	.	.	1	1
	Negative	.	2	.	.	0	2
Cm, Tc	Positive	.	.	.	.	2	2
	Negative	.	.	.	.	0	0
Sm	Positive	.	0	0	.	.	0
	Negative	.	3	3	.	.	6
Cm	Positive	1	.	.	.	1	2
	Negative	0	.	.	.	0	0
Tc	Positive	.	2	4	.	7	13
	Negative	.	2	2	.	0	4
	Total (Strains)	15	25	17	2	24	83

strains irrespective of their antibiogram. In contrast, of 25 *Sh. flexneri* 2a strains transfer was positive in only 6 and of 17 *Sh. flexneri* 3 strains in only 8. (In both cases the χ^2 method indicated significant differences.)

As a rule the frequency of transfer varied from 10^5 to 10^7 and, considering the most frequent R -groups no notable difference was demonstrable for any of the serotypes (Table III).

3. *Treatment with acridine dye.* Another criterion of the episomic nature of antibiotic resistance is the deletion by treatment with acridine dyes. Sixty-two of our resistant Shigella strains were carried through one passage in broth containing 50 μg/ml of acridine orange. Fourty-four of these strains were pre-

Table III

Distribution of transfer frequency of some R groups in relation to antigenic structure

	Sm, Cm, Tc			Sm, Cm			Tc		
	10^5	10^6	10^7	10^5	10^6	10^7	10^5	10^6	10^7
Sh. flexneri 1b**	1	6	4	.	.	1	.	.	.
,, 2a	2	1	.	.	.	1	2	.	.
,, 3	1	2	3	.	.	.	.	2	2
,, 4a	.	.	.	.	2	.	.	.	.
Sh. sonnei	1	4	1	1	4	1	1	3	2

Transfer frequency = successful transfer/original donor cell; Sm = streptomycin-resistant; Cm = chloramphenicol-resistant; Tc = tetracycline-resistant.
** No. of strains

viously subjected to transfer (conjugation) experiments. Twelve of them were and 32 were not able to transmit their resistance factor. The remaining 18 strains were phage-resistant Shigellae unsuitable for transfer experiments. In only 4 out of the examined 62 Shigella strains did the elimination of drug resistance fail. Out of these 4 strains, one Tc-resistant *Sh. flexneri* 3 strain gave a negative result also in transfer experiments. Thus its resistance was not episome-mediated. The other 3 were all *Sh. sonnei* strains, one Sm, Cm and two Tc-resistant.

Results of acridine orange deletion in relation to the antigenic structure and antibiograms of the examined strains are summarized in Table IV.

Table IV

Acridine deletion of R-factor in relation to the antigenic structure and antibiogram

	Sm, Cm, Tc	Sm, Cm	Sm, Tc	Cm, Tc	Sm	Cm	Tc
Sh. flexneri 1b	4 *(17%)**	1 (2%)	.	.	.	.	1 (2%)
,, 2a	9 (21%)	3 (61%)	3 (17%)	.	3 (60%)	1 (8%)	.
,, 2b	2 (7%)	.	.	.	.	.	.
,, 3	5 (40%)	1 (2%)	.	.	3 (41%)	.	3 (3%)
,, 4a	.	1 (1%)	.	.	.	.	.
,, var. X	.	.	.	.	.	1 (1%)	.
Sh. sonnei	7 (50%)	7 (27%)	.	1 (3%)	.	.	2 (10%)
Sh. boydii 12	1 (5%)	.	.	.	.	.	.

* strains
** The percentual values represent the average ratio of the elimination of resistance as related to the number of examined colonies

The presented data do not permit conclusions as to the possible relationship between the success of elimination and the antigenic structure or the type of R-factor. The wide variation of the efficacy of elimination (from 0.1 to 100 per cent) is, however, suggestive of its strain-dependence.

4. *Segregation.* In our material segregation of the R-factors was frequently encountered in both transfer and acridine orange elimination experiments. Table V shows the pattern of segregation of Sm, Cm, Tc-resistance in acridine experiments.

Table V

Segregation of antibiotic resistance in acridine elimination experiments
1. Segregation of Sm-, Cm-, Tc-resistant strains

Designation of strain	Segregation					
	Sm, Cm, (Tc)	Tc	Sm	(Sm), Cm, Tc	Sm, Tc, (Cm)	Cm
9			8/112	7/112		
41			6/17	5/17		
26					8/18	12/18
5			2/27	18/27		
24			9/44	2/44		
10	0/128	113/128				
52			57/154	150/154		
68	7/27	13/27				
64					1/45	22/45
70	2/30	5/30				
88					2/111	8/111
KP	1/20	4/20				
636	4/23	0/23				
379	5/82	7/82				
366			5/95	3/95		
732	6/84	3/84				
155					0/46	1/46
926					7/118	31/118

The numerator shows the number of colonies losing resistance, the denominator the number of examined colonies. Against the antibiotics put in brackets, resistance was in most cases not verified since colonies having lost resistance were not further examined, observations being performed on media containing the appropriate antibiotic.

Of the examined 28 Sm-, Cm-, Tc-resistant strains 18 exhibited segregation of R-factors upon treatment with acridine orange. The proportion of strains losing only Tc-, Sm- or Cm-resistance was roughly similar (7 : 6 : 5). Also the extent of segregation was variable, being obviously dependent on the

strain respectively the R-factor. For example, the *Sh. flexneri* 3 strain designated "10" segregated exclusively in loss of Tc-resistance.

Out of the 13 Sm-, Cm-resistant Shigella strains 7 exhibited segregation. In 4 of them, elimination of the R-factor was never complete as only loss of Sm-resistance could be observed.

In transfer experiments segregation was more often detectable on the basis of differences in transfer frequency. From 21 examined Sm-, Cm-, Tc-resistant Shigella strains 20 produced a R-transfer suggestive of segregation. A positive or negative trend of segregation of Tc-, Sm-, and Cm-resistance factors was observed with 7, 9 and 3 strains, respectively (Table VII).

Table VI

Segregation of antibiotic resistance in acridine elimination experiments
2. Segregation of Sm-, Cm-resistant strains

Designation of strain	Segregation	
	Sm	Cm (Sm)
14	55/134	0/270
27	20/72	0/163
33	18/63	2/63
63	78/201	0/398
32	118/125	122/125
58	54/58	56/58
421	6/87	0/87

The numerator shows the number of colonies losing resistance, the denominator the number of examined colonies. Against the antibiotics put in brackets, resistance was in most cases not verified since colonies having lost resistance were not further examined, observations being made on media containing the appropriate antibiotic.

Tc-resistance segregated exclusively in positive direction, Cm-resistance exclusively in negative direction, whereas Sm-resistance in 1 case in positive and in 8 cases in negative direction.

Of ten Sm-, Cm-resistant Shigella strains analysed by transmissibility tests all exhibited segregation of resistance factors (Table VIII).

In every case, the tendency of segregation was determined by the greater independent transfer of Cm-resistance. Transmissibility of Sm-resistance was often not demonstrable [8].

5. *Detailed study of some representant Shigella strains.* Detailed examinations were carried out with two Sm-, Cm-, Tc-resistant, four Sm-, Cm-resistant and five Tc-resistant Shigella strains. The effect of the elimination of drug-resistance with acridine dye, including segregation, was compared in 1 and 10 passages. The effect of serial cultivation in broth (1 and 10 passages) on spon-

taneous loss of the R-factor was examined and the transferability of R-factors was tested. By replica plating from plain agar we isolated some colonies that had lost resistance; the R^+ *Serratia marcescens* colonies were obtained in transfer experiments. The antibiotic resistance of the R^+ and cured lines was tested by qualitative and quantitative assay.

Table VII

Segregation of antibiotic resistance in transfer experiments
1. Segregation of Sm-, Cm-, Tc-resistant strains

Designation of strain	Sm, Cm (Tc)	Transfer frequency				
		Tc	Sm	Cm, Tc (Sm)	Sm, Tc (Cm)	Cm
9			10^7	10^6		
41			10^6	10^7		
157			—	10^7		
P	—	10^5				
932			—	10^5		
Bö			10^7	10^6		
30	—	10^7				
53					10^6	—
56	—	10^7				
57					10^5	10^6
59	—	10^6				
71			10^7	10^5		
75			10^7	10^5		
76			10^7	10^5		
77	—	10^7				
78	—	10^7				
79					10^6	—
82			—	10^6		
967	—	10^6				

Segregation was estimated on the basis of difference in transfer frequency.
Resistance to antibiotics in brackets has not been verified in most cases.
— = transfer frequency $< 10^7$

The effect of acridine treatment, and broth passages on certain representatives are shown in Table IX.

After one passage in acridine orange containing medium the Sm-, Cm-, Tc-resistant strain "9" produced a greater ratio of segregation in loss of Sm-resistance alone than in loss of Sm-, Cm-, Tc-resistance (R^-). Ten passages in the same medium resulted in complete loss of Sm-, Cm-resistance. Elimination of the complete R-factor occurred in about 37 per cent. The frequency of the

spontaneous loss of R-factor was notable, it being completely eliminated after 10 passages. The Sm-, Cm-, Tc-resistant strain "14" exhibited a similar behaviour. In the case of the four Sm-, Cm-resistant strains acridine elimination of drug resistance resulted in the loss of Sm-resistance alone, in both 1 and 10 passages. Only one of these strains produced two colonies which lost the complete R-factor. The spontaneous loss of the R-factor occurred in the opposite direction. In two cases only Cm-resistance was lost, but this one completely,

Table VIII

Segregation of antibiotic resistance in transfer experiments
2. Segregation of Sm-, Cm resistant strains

Designation of strain	Transfer frequency	
	Sm. (Cm)	Cm
14	10^7	10^6
27	10^7	10^5
33	—	10^6
63	—	10^5
90	—	10^5
13	—	10^7
34	—	10^6
45	—	10^6
60	—	10^7
73	—	10^7

Segregation was estimated on the basis of difference in transfer frequency.
Resistance to the antibiotic in brackets has not been verified in the majority of cases.
— = Transfer frequency $< 10^7$

in another case (strain 33) there was a substantial loss in Cm-resistance, whereas with strain 27 complete loss of Sm-resistance and a lesser but substantial loss of Cm-resistance was observed.

Two of the examined Tc-resistant strains (35 and 18) lost drug resistance but only partly after one passage but completely after ten passages, in acridine orange containing medium. In these strains no spontaneous loss of the R-factor was demonstrable. In contrast, the Tc-resistant strain No. 42 retained its drug-resistance during acridine orange treatment, but produced spontaneously some R^- colonies after 10 passages in broth.

The level of resistance determined by the R-factor was estimated by the quantitative antibiotic-sensitivity test (Table X).

As shown in Table X, transfer of the R-factor between Shigella and Serratia resulted — irrespective of segregation — in the same level of resistance in the recipient as had been carried by the donor.

Table IX

Loss and segregation of R-factor in Shigella strains passaged in broth and treated with acridine on one and more occasions

Designation of strain	Acridine-treatment		Cultivation in broth	
	1 passage	10 passages	1 passage	10 passages
9 Sm. Cm. Tc	Sm 8/115* Sm. Cm. Tc 3/115	Sm. Cm 80/80 Sm. Cm. Tc 30/80	Sm 12/92	Sm. Cm. Tc 70/70
41 Sm. Cm. Tc	Sm 18/51 Sm. Cm. Tc 15/15	Sm. Cm. Tc 29/29	Sm 2/79	Sm. Cm 27/63 Sm. Cm. Tc 22/63
14 Sm. Cm	Sm 55/134	Sm 6/32	Sm 30/79	Cm 250/250
27 Sm. Cm	Sm 20/72	Sm 2/13	—	Cm 88/93 Sm 93/93
33 Sm. Cm	Sm 16/33 Sm. Cm 2/63	Sm 36/36	Sm 11/105	Cm 123/123 Sm. Cm 2/122
63 Sm. Cm	Sm 73/201	—	—	Cm 125/125
35 Tc	1/21	94/94	0/120	0/154
18 Tc	1/104	45/45	0/142	0/112
42 Tc	0/210	0/95	0/85	9/123

* The numerator is the number of colonies having lost resistance, the denominator is the number of the examined colonies. Segregation is indicated by the incongruence of the antibiogram with the original one.

Another observation has been made with segregants upon acridine elimination of resistance. The colonies of both the Sm-, Cm-, Tc-resistant strains 9 and 14 were, upon loss of the complete R-factor resistant exclusively to 15.62 μg Sm, whereas segregants having lost Sm-resistance alone to 62.5 μg of Sm. The

R^- colonies of Sm-, Cm-resistant strain 33 were resistant to 7.8 μg of Sm, whereas its Sm^- segregants to 62.5 μg of Sm.

Table X

Quantitative study of the antibiotic resistance (R-factor) of some representative Shigella strains after acridine

Designation of strain	Resistance								
	Original resistance			After elimination			Mediated by transfer*		
	Sm	Cm	Tc	Sm	Cm	Tc	Sm	Cm	Tc
9	250**	500	500	62	.	.	.	500	500
Sm. Cm. Tc				15	2	3	250	500	500
41	1000	500	500	62	.	.	1000	.	.
Sm. Cm. Tc				15	2	1	.	500	500
14	1000	500	—	62	—	.	500	500	—
Sm. Cm									
27	500	500	—	62	.	—	500	500	—
Sm. Cm									
33	250	500	—	62	.	—	250	.	—
Sm. Cm				8	2	—	250	250	—
63	500	500	—	62	.	—	500	500	—
Sm. Cm									
35	—	—	250	—	—	4	—	—	250
Tc									
40	—	—	250	—	—	—	—	—	500
Tc									
18	—	—	500	—	—	8	—	—	.
Tc									
42	—	—	500	—	—	.	—	—	500
Tc									
16	—	—	250	—	—	8	—	—	.
Tc									

* = *Serratia marcescens*
** = μg/ml

Incidence of episomal resistance among Shigella strains. Results obtained with two methods (transfer and deletion of R-factor) verified the episomic nature of drug-resistance in 102 of 103 Shigella strains representing different serotypes and various types of single or multiple resistance. Evidence of the episomic nature of resistance was lacking in one case only, a Tc-resistant *Shigella flexneri* 3 strain.

Table XI

Summarized results concerning episomic antibiotic resistance of Shigella strains

Antibiogram	Number of strains*	Transfer experiment		Acridine elimination		Resistance in the two tests	
		Positive	Negative	Pos.	Neg.	Episomic	Not verified
Sm. Cm. Tc	48	24	13	28	0	48	0
Sm. Cm.	18	10	6	13	0	18	0
Sm. Tc	4	1	2	3	0	4	0
Cm. Tc	3	2	0	1	0	3	0
Sm	6	0	6	6	0	6	0
Cm	5	2	0	2	1	5	0
Tc	19	12	5	5	3	18	1
Total	103	51	32	58	4	*102*	*1*

Sm = Streptomycin-resistant; Cm = Chloramphenicol-resistant; Tc = tetracycline-resistant.

Discussion

As already mentioned, the unexpectedly high incidence of multiple resistant Shigella strains induced Japanese authors to advance the theory of the non-mutational origin of resistance. The first classical Sm-, Cm-, Tc-, Sa-(sulphonamide) resistant strains were isolated by KITAMOTO [4]. Shortly afterwards AKIBA [5] presented evidence for the transmissibility of antibiotic resistance. MITSUHASHI *et al.* [6] and simultaneously WATANABE and FUKUSAWA [7] arrived at the conclusion that between different genera of Enterobacteriaceae antibiotic resistance is transmissible by conjugation mechanism. MITSUHASHI *et al.* [8, 9] observed spontaneous loss of resistance and its elimination by treatment with acridine dyes. On the basis of the above observations the determinant of transmissible resistance has been considered to be episomic [7]. The genetic information responsible for episomic resistance was termed R-factor by ISEKI [7]. The resistance determinants carried by the R-factor allowed the differentiation of several "R"-factors; MITSUHASHI [11] for example reported on the isolation of the following R-factors: (1) Sa. Sm. Cm. Tc; (2) Sa. Sm. Cm; (3) Sa. Sm; (4) Sa. Cm; (5) Cm. Tc; (6) Sm; (7) Tc and (8) Cm, isolated as segregant of a (R) Sm-, Cm-resistant strain.

The present studies had been prompted by the remarkably high incidence of multiple resistant Shigella strains in Hungary. The occurrence of episomic resistance among these strains was studied by examing about 100 Shigella strains of various serological types and antibiograms.

Transfer experiments were performed using as recipient the sensitive *Serratia marcescens* strain 229, isolated in the course of routine examinations.

Successful transmission of the R-factor between Shigella and Serratia was already shown by FALKOW *et al.* [12]. In our experiments the incidence of transmissibility amounted to 61 per cent. Our observation of the apparent relationship of antigenic structure with transfer frequency warrants further studies. With *Sh. sonnei* strains, resistance was transmissible in nearly every case (23 out of 24), whereas with *Sh. flexneri* 2a and 3 strains transfer frequency was significantly lower, 6/25 and 8/17, respectively. The fact, however, that strains unable to transfer resistance by conjugation gave positive results in elimination experiments with acridine orange, opposes the concept attributing the phenomenon to the chromosomal fine structure of *Sh. sonnei* or — in other words — to its adjuvant action on the integration of the R-factor. Hence we are inclined to accept in these cases the existence of mutant R-factors carrying defective RTF [10].

In the course of the present study, segregation of the R-factor was observed not only in transfer but also in deletitive experiments. Segregant R-factors occurred more often than non-segregant ones; in this report we have, however, restricted ourselves to data on the segregation of the two predominant types of multiple resistance, Sm. Cm. Tc. and Sm. Cm (see Tables V and VIII).

Inconsistently with the concept excluding the incidence of segregation during acridine treatment [13], we observed this phenomenon with the greater part of the examined strains. The tendency of segregation was found to be congruent in transfer and elimination experiments. For example, in elimination experiments mostly the Sm-resistance of Sm-, Cm-resistant strains was lost, whereas in transfer experiments Cm-resistance showed a greater transmissibility.

With the applied technique, definite conclusions as to segregation could be derived only from the frequency of transfer, differing greatly among individual antibiotics. Smaller differences may result also from methodical errors, but differences attaining two orders of magnitude can safely be interpreted as segregation.

According to literary data the mechanism of segregation is non-uniform. WATANABE and FUKUSAWA [14] noted that genetic information may be exchanged several times between R-factor and host cell chromosome. MITSUHASHI *et al.* [15] presented experimental evidence of the presence of several R-factors within one and the same cell. This relatively unstable condition results in a relatively highy frequency of segregation. The data concerning the quantitative determination of the resistance of segregants (Table X) are supportive of the latter mechanism.

In the same series of experiments including but few strains the spontaneous loss of the R-factor was also examined and correlated with the results of acridine-induced elimination of resistance transfer. Sm-segregants were demonstrable after one passage in broth already. Upon acridine treatment, Sm-,

Cm-resistant strains lost mainly their Cm-resistance. These findings correlate well with the observations of LEBEK [16] concerning the relative stability of Tc-resistance.

Our results indicate that the majority of the Shigella strains isolated in Hungary are carrying episomic resistance irrespective of their antigenic structure or antibiogram. This would explain the rapidly increasing incidence of antibiotic resistant strains, particularly of multiple resistant ones. Our observations have yielded additional proof of the world-wide incidence of R-factors.

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STUDIES OF THE RELATIONSHIP BETWEEN LIPID CONSTITUENTS AND BIOLOGICAL PROPERTIES OF PROTEUS BACTERIA

By

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Summary. Total lipid and phosphatide content and fatty acid spectrum of 5 different Proteus strains *(Proteus vulgaris, P. mirabilis, P. morgani, P. rettgeri* and *P. inconstans)* have been examined.

(1) Total lipid content of Proteus strains varied from 5.5 to 7.5 per cent, their phosphatide content, from 4 to 4.9 per cent.

(2) All the examined strains contained four phosphatide components *viz.* cardiolipin, cephalin, lecithin and lysocephalin; the quantitative relationships of these components varied with the individual strains.

(3) The lipid components of the examined Proteus strains are built up of essentially the same fatty acids. The bulk is constituted by myristic acid, beta-hydroxy-myristic acid, palmitic acid, and fatty acids C_{17} and C_{19} containing cyclopropane ring.

(4) There is a conspicuous prevalence of long-chain fatty acids and special fatty acids, whereas unsaturated fatty acids are present in low quantities.

(5) Conclusions are drawn concerning the relationship of the quality and quantity of bacterial lipids with the bacterial membrane permeability, resp. resistance.

The biological role of bacterial lipids, the lipid constituents of the various bacteria as well as their changes under external influences are not quite clear. A closer approach of the problem has been made possible only by recent analytical methods.

In the present experiments the total lipid and phospholipid content and fatty acid range were determined in 18-hour cultures of one strain each of 5 biologically different subgroups of Proteus, in order to elucidate whether the composition of bacterial lipids had a relationship with certain biological properties, *e.g.* resistance.

Materials and methods

Strains, P. vulgaris (61001), *P. mirabilis* (60007), *P. morgani* (63001), *P. rettgeri* (65002), *P. inconstans* (67001). Figures in brackets indicate register numbers of the collection of the State Institute of Hygiene, Budapest.

Culturing was carried out in the following semisynthetic media. Medium (g/1000 ml): Lipid-free casein hydrolysate — DIFCO 10; sodium hydrochloride 5; disodium hydrophosphate 5; potassium dihydrophosphate 5; ferro ammonium sulphate 0.03; magnesium sulphate 0.01; manganese sulphate 0.01; distilled water ad 1000 ml. The medium was precisely adjusted to pH 7.2. All chemicals used were of analytical grade.

Culturing conditions. The bacteria were cultivated for 18 hours in shaken cultures at $37° \pm 0.1°$ C in water bath, in 500 ml Erlenmeyer flasks each containing 150 ml nutrient medium. Shaking rate was 120/min.

Extraction of bacterial lipids, analysis of phosphatides and identification of fatty acids was carried out as described previously [1].

Results

The lipid content of bacteria depends to a certain extent on the age of the culture. In old cultures lipid concentration is as a rule high, hence only cultures of identical age can be compared as to lipid content.

In the first series of experiments we examined the phase of growth after culturing for 18 hours. Multiplication was registered by a biophotometer (Fig. 1).

Fig. 1 shows no notable difference in growth rate between the examined 5 strains. All of them were through the lag phase by the 6th hour, thus in the 18th hour they were in the late stac. phase. *Proteus rettgeri* formed rough colonies

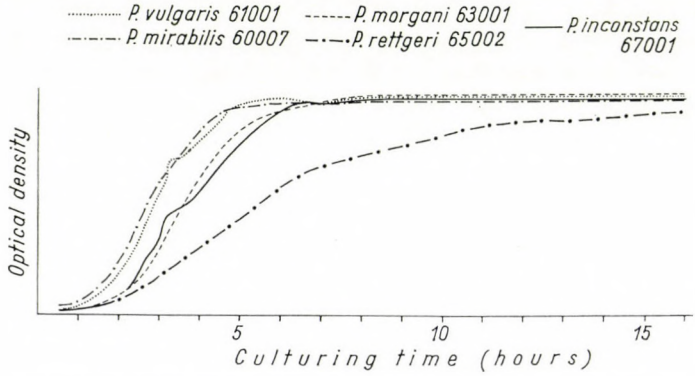

*Fig. 1.*Growth of *B. proteus* strains as measured by the Bonet-Maury et Jouan biophotometer. The growth curve was determined at 37° C. Instead of shaking an electromagnetic mixer was used. Multiplication started about 2 hours earlier than in shaken cultures

scattered irregularly over the surface of the medium. Thus its growth curve was flatter than, but its total germ count essentially similar to, that of the other 4 types.

Table I shows the total lipid and phosphatide content per dry material.

Table I

Lipid content of different B. proteus strains, in per cent of the dry material content

Organism	Pure total lipid content	Phosphatide content
P. vulgaris	5.5	4.9
P. mirabilis	6.2	4.0
P. morgani	5.6	4.2
P. rettgeri	6.6	4.1
P. inconstans	7.5	4.8

Table I reveals that the total lipid content of the strains varied from 5.5 to 7.5 per cent, their phosphatide content from 4.0 to 4.9 per cent. The phosphatide content of *P. vulgaris* was conspicuously high, amounting to more than 80 per cent of the total lipid content. With less pathogenic strains, phosphatides amounted to approximately two thirds of the total lipid content.

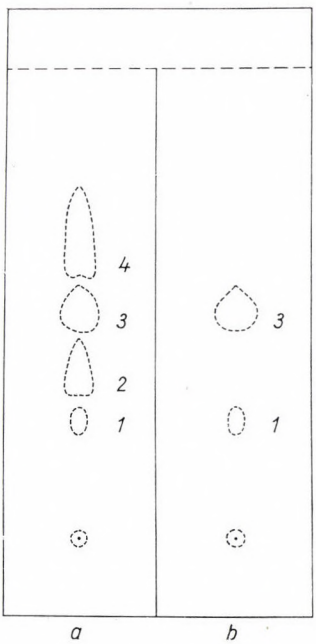

Fig. 2. Thin layer chromatogram of the phosphatides of *P. vulgaris*. Layer: Silicagel G, 500 μ. System: chloroform-methanol-water 65 : 25 : 4. Part(a) of the chromatogram was developed with 5 per cent alcoholic phosphomolybdenic acid, part(b) with nynhydrin. Components: (1) Lysocephaline; (2) Lecithine; (3) Cephaline; (4) Cardiolipin

Fig. 2 presents the thin-layer chromatogram of the phosphatides of *P. vulgaris*.

In the chromatogram the phosphatides of *P. vulgaris* were separated into four constituents. The first spot was probably lysocephaline, as it gave a positive reaction with phosphomolybdenic acid and nynhydrin. The second spot, reacting positively with phosphomolybdenic acid and Dragendorff's reagent, was identified as lecithin. The third spot reacted with phosphomolybdenic acid and nynhydrin and was identified as cephaline. The fourth spot gave a colour reaction with phosphomolybdenic acid and was thus considered cardiolipin.

Identification of the spots with control also showed the phosphatides to consist of cardiolipine, cephaline as the main components and in lesser amounts

of lecithin and lysocephaline. Fig. 3 shows the densitometric evaluation of thin layer chromatograms.

The five strains differed only in the quantity of their phosphatide constituents. All bacteria of the Proteus group contain the same phosphatides.

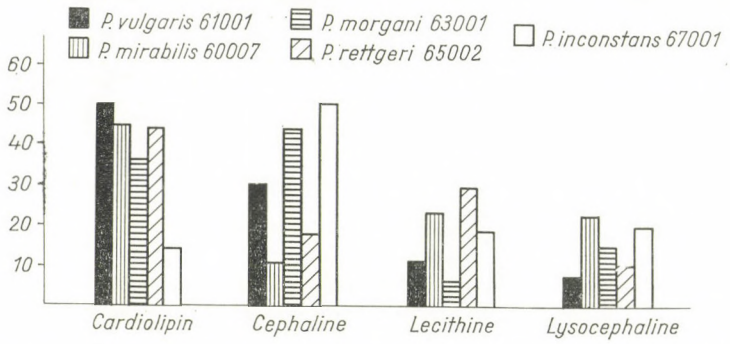

Fig. 3. Percentual composition of phosphatide constituents of Proteus strains. To a Mod. 425 Photovolt densitometer a frame suited to hold a 3 by 30 cm glass slide was fitted. The slides were forwarded mechanically. 500 μg samples, 500 μ layer, 2.5 per cent phosphomolybdenic acid. Zero point was adjusted without filter in the unstained area between cardiolipin and front

In the next series the fatty acid components of the various Proteus strains were determined.

Analysis of the fatty acid spectrum of the different Proteus strains revealed that in each of them the lipid part consisted of essentially the same fatty acids, of which the five main components amounted to more than 90 per cent of the total fatty acid component.

The fatty acid spectrum of *P. vulgaris* is presented in Fig. 4.

Table II

Percentual distribution of fatty acid components in different B. proteus strains*

Fatty acids Name	Abbreviated sign	*P. vulgaris*	*P. mirabilis*	*P. morgani*	*P. rettgeri*	*P. inconstans*
Myristinic acid	C_{14}	14	15	15	22	22
Beta-hydroxymyristinic acid	β-OH C_{14}	8	5	4	8	6
Hexadecanoic acid	$C_{16:1}$	2	1	2	9	21
Palmitic acid	C_{16}	39	36	35	34	38
Methylenehexadecanoic acid	$C_{17\Delta}$	19	20	25	18	1
Octadecanoic acid	$C_{18:1}$	1	2	2	4	4
Methyleneoctadecanoic acid	$C_{19\Delta}$	11	15	2	—	—
Other fatty acids total:		6	6	15	5	8

* Percentual data obtained by beta-ray ionisation detector, without calibration factors

As shown in Fig. 4, the bulk of the fatty acid of *P. vulgaris* consists of 5 fatty acids: myristinic acid, beta-OH-myristinic acid, palmitic acid and C_{17} and C_{19} fatty acids with cyclopropane ring.

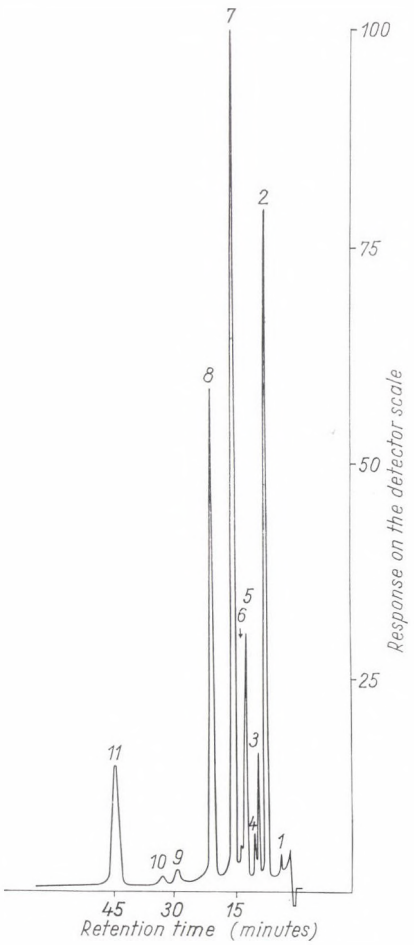

Fig. 4. Gas chromatogram of the fatty acid components of *P. vulgaris* (61001). Pye Argon Chromatograph; flow rate, 60 ml/min.; Temperature, 60° C; Detector voltage, 1250 V. Sample, 0.1 μl concentrated fatty acid methylester. Column: 10 per cent Apieson L fat, Celite-545, 100—120 mesh

Peaks: 1 = lauric acid; 2 = myristinic acid; 3 = n-pentadecanoic acid; 4 = ante-iso-pentadecanoic acid; 5 = beta-hydroxymyristinic acid; 6 = hexa-decanoic acid; 7 = palmitic acid; 8 = methylene-hexadecanoic acid; 9 = octadecanoic acid; 10 = stearinic acid; 11 = methylene-octadecanoic acid

The fatty acid components of the five different Proteus strains is shown in Table II.

Thus the above strains differ not so much in the composition as in the quantitative relationships of their fatty acid constituents.

The similarity of the fatty acid spectra of the examined Proteus strains is indicative of their phylogenetic relations. *P. vulgaris*, *P. mirabilis* and *P. morgani* strains contain a remarkably great amount of cyclopropane fatty acids. Their quantity attains or approximates one third of the total fatty acid content, while unsaturated fatty acids were present in small amounts.

Discussion

An obvious relationship was demonstrable between the total lipid content of the different bacterium species and their main biological properties. For example, species rich in lipids displayed a higher resistance to certain external influences, disinfectants and antibiotics than those with lower lipid content [2]. The high lipid content of the highly resistant Mycobacteria is well-known. The role of lipids in the virulence, toxicity and pathogenic action of bacteria must not be disregarded either.

In the present experiments a bacterium group of medium virulence, the Proteus group was examined for total lipid content, quantity and composition of phosphatides and fatty acid spectrum. The examinations were carried out on one member each of five Proteus subgroups (*P. vulgaris*, *P. mirabilis*, *P. morgani*, *P. rettgeri*, *P. inconstans*).

Among the Enterobacteriaceae the Proteus group is the most resistant to untoward influences and least sensitive to antibiotics; their lipid content, inclusive of phosphatide constituents, is also the highest. While in both chloramphenicol sensitive and resistant *E. coli* the total lipid content amounts to 3.9 to 4.2 per cent, including 1.0 to 2.3 per cent phosphatides [3], bacteria of the Proteus group contain 5.5 to 7.5 per cent of the total lipid, including 4 to 5 per cent phosphatides. The still more resistant species *Ps. pyocyanea* was found to contain more than 5 per cent phosphatides and about 8 per cent total lipid [1].

Phospholipids are known to take part in the structure of the cell membrane's double lipid layer. Thus, the phospholipid content of the bacterial cell may be considered an indicator of the volume and differentiation of the cell membrane's inner structure.

The importance of phospholipids as structural elements of the cell is due to their special physical and chemical properties, while, as dynamic cellular components they have a decisive role in the transport of certain materials and ions.

The relationship between the bacterial membrane's lecithin and cephalin content and its permeability is obviously demonstrated. The high phospholipid

content of the Proteus group correlates well with the high resistance of these bacteria.

Analysis of the fatty acid spectrum of Proteus bacteria revealed the prevalence of long-chain and large amount of cyclopropane fatty acids, and also the low amount of unsaturated fatty acids. This constitution is closely related with the cell membrane's stability and permeability and hence with the cell's resistance. KÖGL *et al.* [4] and WALKER and KUMMEROW [5] have shown that on increasing the unsaturated fatty acids in the erythrocyte membrane, this becomes more permeable and less stable. Glycerophosphatides containing unsaturated fatty acids form a looser pattern in the biomolecular membrane layer, and are thus more readily dissociable than those containing saturated fatty acids. In addition, the longer the hydrocarbon chain, the higher the adhesion resulting from the interaction of CH_2 pairs. As a result, the membrane becomes rigid and less permeable both structurally and functionally. Hence, lipids containing long-chain saturated or cyclopropane fatty acids reduce membrane permeability and thus increase the resistance of the bacterial cell. According to certain authors' and our own observations, in certain bacteria the quantity of cyclopropane fatty acids increases parallel with the ageing of the culture [1]. Simultaneously, its resistance tends to increase. The actual function of cyclopropane-ring fatty acids is not yet clear, but they appear to have favourable physical properties, their melting point lying between those of the saturated and unsaturated fatty acid pairs.

Substitution of unsaturated by cyclopropane-ring fatty acids in the phospholipid molecule does not alter the latter's ability to form soluble lipid mycelia, but stabilizes the membrane's structure and thus increases the cell's resistance. Our examinations indicate that the main difference between sensitive and polyresistant *Staphylococcus aureus* strains lies in their cyclopropane fatty acid content which is much higher in the resistant strains. Such fatty acids are absent in highly antibiotic-sensitive bacterial species, e.g. *Streptococcus* and *Treponema pallidum*. Hence the development of increasing resistance by Proteus bacteria necessarily infers the role of long-chain fatty acids as well as the absence of unsaturated fatty acids.

The identity of the fatty acid constituents of these bacteria is suggestive of their phylogenetic relationship. Considering that not only the fatty acid spectrum but also the phospholipid components of *P. inconstans* are identical with those of the other Proteus types, its classification with the Proteus group seems to be supported also by biochemical facts.

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EFFECT OF TANNIC ACID ON DIFFERENT VIRUSES

By

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Summary. The action on different viruses of an aqueous solution of commercial tannic acid has been examined. *Herpesvirus hominis*, *Herpesvirus suis* and type 3 parainfluenza virus were found to be sensitive while poliovirus types 2 and 3, and adenovirus types 3, 4 and 6 to be resistant to tannic acid. The action of tannic acid appeared to be virucidal. Tannic acid added to cells infected with *Herpesvirus hominis* enhanced rather than inhibited viral multiplication. Cells treated with tannic acid prior to infection with herpesviruses exhibited a decreased sensitivity to the agent. The antiviral activity of tannic acid is supposed to be related to the polyanionic nature of the compound.

During recent years a number of plant extracts has been examined for virus inhibitory substances and several of them were found to inactivate different animal, plant and bacterial viruses [1—7]. In the course of such studies tannic acid was shown to have a certain antiviral effect [8—12].

In this laboratory the virucidal effect on *Herpesvirus hominis* was demonstrated of aqueous extracts of dry oak and beech leaves [13]. The fact that oak-leaf extracts gave a tannic acid specific reaction with $FeCl_3$ prompted us to examine the antiviral effects of tannic acid.

Materials and methods

Tissue cultures and media. HeLa cells were grown in GEY's medium containing 5 per cent rabbit serum and 0.25 per cent lactalbumin hydrolysate.

Chicken cell cultures were prepared from 11-day old chick embryos. Cells were obtained by trypsinisation. After appropriate fractional centrifugation dishes 50 mm in diameter were seeded with 25×10^6 cells each. The medium consisted of GEY's solution with 4 per cent Tris buffer (pH 7.6), 5 per cent calf serum and 0.25 per cent lactalbumin hydrolysate.

Virus strains. Herpesvirus hominis, adenovirus types 3, 4 and 6, poliovirus types 2 and 3 were used, all grown on HeLa cells.

Herpesvirus suis was grown on chick embryo fibroblasts according to a previously described method [14].

Virus titration. Viruses grown on HeLa cells were titrated in HeLa tube cultures by infecting 3 parallel tubes each with successive 10fold viral dilutions. Titres were expressed as $TCID_{50}$ according to the formula of REED and MUENCH [15]. Results were read on the 6th day of incubation with poliovirus and *Herpesvirus hominis*. Titres of type 3 parainfluenza virus were determined by the haemadsorption test after 6 days of incubation using a 0.4 per cent suspension of guinea pig erythrocytes.

Cultures infected with adenoviruses were examined on the 24th day of incubation. To maintain cells in good condition the medium was changed every 4 to 5 days.

Titration of *Herpesvirus suis* was performed by the plaque method in chicken fibroblast monolayers as described earlier [16]. This method was a modification of PORTERFIELD's [17] technique.

Tannic acid solution. The tannic acid preparation used had an absorption maximum at 280 mμ wavelength at 20 μg/ml concentration. The stock was a 200 μg/ml tannic acid solution in saline. The solution was sterilized by filtration through a fritted glass filtre G-5 (Jena).

Examination of virucidal effect. Equal amounts of an appropriate tannic acid solution were added to each of a tenfold dilution series or to an undiluted sample of the virus tested. The mixtures were incubated at room temperature (20—25° C) for 2 hours. At the end of the above incubation period, mixtures with serial virus dilutions were inoculated in 0.2 ml volume each into HeLa cell cultures. Mixtures with undiluted virus were, however, serially diluted in tenfold steps prior to inoculation. Control cultures were prepared with identical amounts of saline.

Herpesvirus suis was diluted so as to contain 10^3 to 10^4 PFU/ml in Hanks' balanced salt containing 10 per cent of pH 7.6 Tris buffer. Equal amounts were mixed of the above virus suspension and of the tannic acid solution. After appropriate incubation 0.2 ml of the undiluted or 1 to 10 diluted mixture were transferred to chicken fibroblast monolayers in Petri dishes. After 2 hours incubation at 35° C the infected monolayers were overlaid with agar.

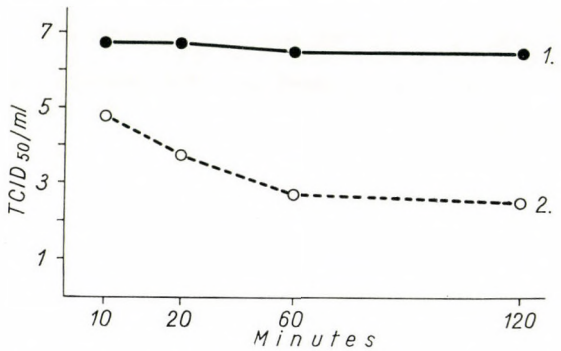

Fig. 1. Inactivation of *Herpesvirus hominis* by tannic acid. 1 : control; 2 : tannic acid treated

Examination of virucidal effect in mice. Identical amounts of tannic acid solution were mixed with a series of tenfold dilution (10^{-1} to 10^{-6}) of *Herpesvirus hominis* and the mixtures incubated for 2 hours at room temperature. The material was then inoculated intracerebrally in 0.03 ml doses to albino mice of 25 to 30 g body weight. Animals receiving a virus plus saline mixture served as controls.

Examination of virostatic effect. HeLa cell cultures were infected with appropriate dilutions of *Herpesvirus hominis* and incubated for 2 hours at 35° C. The virus was then removed and the cultures washed 3 times with Hanks' solution. The washed cultures received a 20 μg/ml tannic acid containing medium and were further incubated at 35° C. Control cultures were prepared with saline instead of tannic acid. The $TCID_{50}$ was determined after 6 days incubation.

Virostatic effect on *Herpesvirus suis* was examined on chicken fibroblast monolayers infected with the virus and washed 3 times with Hanks' solution and finally overlaid with nutrient agar containing different concentrations of tannic acid.

Results

The inactivating effect on *Herpesvirus hominis* is demonstrated in Fig. 1. In these experiments undiluted virus was used.

In the presence of tannic acid a 2 log titre decrease was observed in 10 minutes. In 1 hour a further 2 log drop occurred and at that level the titre appeared to stabilize.

Incubation time at room temperature was always 2 hours in the further inactivation experiments.

In addition to *Herpesvirus hominis*, we examined the effect of tannic acid also on types 3 and 6 of adenovirus, types 2 and 3 of poliovirus and on type 3 of parainfluenza virus. In all these experiments undiluted virus suspensions were used. Results are given in Table I.

Table I

Effect of tannic acid on different viruses grown on HeLa cells

Virus	Titre (log $TCID_{50}$/ml)		Inhibition (log)
	control	tannic acid treated	
Poliovirus type 2	5.74	5.24	0.5
Poliovirus type 3	6.24	6.5	−0.26
Adenovirus type 3	7.5	7.5	0
Adenovirus type 6	7.24	7.74	−0.5
Parainfluenza type 3	6.5	6.24	0.26
Herpesvirus hominis	6.5	2.5	4.0

As shown by the results, *Herpesvirus hominis* appeared to be sensitive, while polio-, parainfluenza- and adenoviruses were resistant to tannic acid at the concentration used.

Results of experiments performed by means of adding tannic acid to different dilutions of the test viruses are presented in Table II.

Table II

Titre decreasing effect of tannic acid added to dilution series of different viruses

Virus	Titre (log $TCID_{50}$/ml)		Inhibition (log)
	control	tannic acid treated	
Poliovirus type 2	5.74	5.5	0.24
Poliovirus type 3	6.5	5.74	0.76
Adenovirus type 3	6.24	6.5	−0.26
Adenovirus type 4	5.24	4.74	0.5
Parainfluenza type 3	6.5	3.5	3.0
Herpesvirus hominis	6.5	2.74	3.76

Even under these conditions, no significant effect of tannic acid was observable on types 3 and 4 of adenovirus and on type 2 poliovirus. The titre difference observed in the case of type 3 poliovirus was not significant either. Parainfluenza virus was, however, just as much sensitive to tannic acid as *Herpesvirus hominis*.

Results of a comparative examination of tannic acid sensitivity of *Herpesvirus hominis* and type 3 parainfluenza virus are given in Table III. This experiment was performed by adding equal amounts of tannic acid solution at different concentrations to the tenfold dilutions of the above viruses.

Table III

Effect of tannic acid at different concentrations on Herpesvirus hominis and type 3 parainfluenza virus

Tannic acid μg/ml	Titre (log $TCID_{50}$/ml)	
	Herpesvirus hominis	Parainfluenza type 3
0	6.50	6.74
0.5	5.50	6.50
1	5.74	6.74
5	4.50	6.24
10	3.50	4.24
50	3.50	3.74
100	2.50	3.50

This experiment, too, showed the sensitivity of type 3 parainfluenza virus to be similar but somewhat lower than that of *Herpesvirus hominis*. Next, the inactivating effect of tannic acid on *Herpesvirus hominis* was examined in mouse inoculation tests. Results are presented in Table IV.

Table IV

Mouse pathogenicity of tannic acid treated Herpesvirus hominis

Inoculum	Titre* (log LD_{50})	Inhibition (log)
Saline + virus	4.74	2
Tannic acid + virus	2.74	

* evaluation on 18th day after infection.

Results given in Table IV show a 2 log decrease of mouse LD_{50} under the effect of tannic acid treatment.

The effect of tannic acid solutions at different concentrations on the plaque forming capacity of *Herpesvirus suis* was studied in chicken embryo fibroblast monolayers. Results are presented in Table V.

Table V

Effect of tannic acid at different concentrations on plaque forming capacity of Herpesvirus suis

Tannic acid μg/ml	Plaque count* (per cent of control)
5	0
2.5	4
1	16
0.5	25
0.25	53
0.1	71
0.05	75
0.01	77
0.005	100

* mean of 4 parallels

The plaque count of a given sample of *Herpesvirus suis* was reduced by about 50 per cent on treatment with a 0.25 μg/ml solution of tannic acid. The average plaque count of the untreated virus was 178.

We studied further the possible virostatic effect of tannic acid on *Herpesvirus hominis* in HeLa and on *Herpesvirus suis* in chicken fibroblast cell cultures. Results of the former experiment are given in Table VI

Table VI

Effect of tannic acid on adsorbed Herpesvirus hominis

No. of experiment	Titre (log $TCID_{50}$/ml)		Inhibition (log)
	Control	Tannic acid treated	
1.	6.50	7.24	−0.74
2.	5.74	7.24	−1.50
3.	5.74	6.50	−0.76

As shown in Table VI, after the adsorption of *Herpesvirus hominis* tannic acid failed to cause a titre reduction; instead, a moderate increase was observed in all the three parallel experiments.

In the case of *Herpesvirus suis* a 20 to 50 μg/ml tannic acid content of the agar overlay failed to cause changes in the plaque count. Thus, none of the examined viruses was sensitive to the effect of tannic acid once adsorption had taken place.

Next it was examined whether tannic acid treatment of the cells would affect their sensitivity to herpesviruses. Results obtained with two different viruses and two different kinds of cell are presented in Table VII.

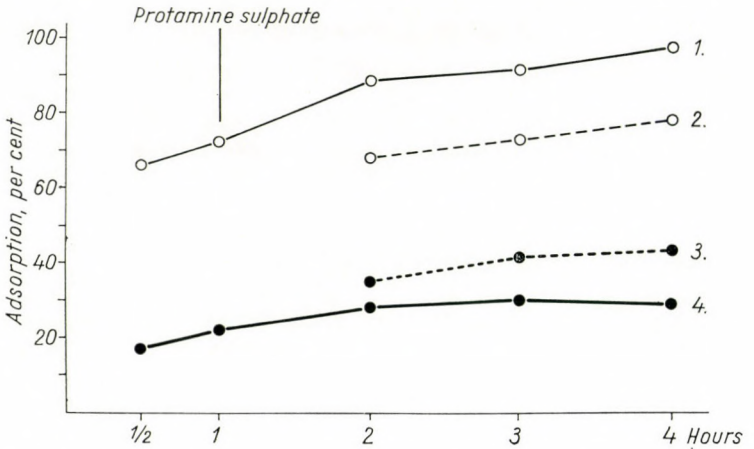

Fig. 2. Effect of protamine sulphate on the adsorption-inhibition of tannic acid on *Herpesvirus suis*. 1 : Virus + Hanks; 2 : Virus + protamine; 3 : Virus + tannic acid + protamine; 4 : Virus + tannic acid

Table VII

Effect of tannic acid on the sensitivity to viruses of HeLa and chicken embryo cells

Cell		Virus	
		Herpesvirus hominis (log $TCID_{50}$/ml)	*Herpesvirus suis* PFU*
HeLa	control	6.50	—
	tannic acid treated	4.74	
Chick	control	—	277
	tannic acid treated		131

* mean of 4 parallels

Tannic acid treatment was found to reduce the sensitivity of both types of cell against the respective herpesvirus.

The reversibility of the tannic acid effect by protamine sulphate was examined using a *Herpesvirus suis* and chicken embryo fibroblast system. Monolayers were infected with a suspension containing 100 PFU of virus and 10 μg of tannic acid. Controls were prepared with virus only. After 1 hour incubation at 35° C, protamine sulphate (50 μg/plate) was added to half of the control and test cultures. To the other half of the control and test cultures saline was given only. At different points of time medium was removed from 2 dishes each of control and test cultures and they were washed 3 times with Hanks' and overlaid with nutrient agar. Results are presented in Fig. 2.

In the presence of tannic acid the plaque count was reduced by about 60 per cent at every point of time examined. Protamine sulphate added at the first hour of adsorption appeared to inhibit further adsorption. The plaque-count-reducing effect of tannic acid was slightly inhibited by protamine sulphate. In the 4th hour of incubation a 15 per cent increase in plaque count was observed in tannic acid and protamine sulphate treated cultures as compared to those treated only with tannic acid.

Discussion

The inhibitory action of tannic acid on tobacco mosaic virus has been known since 1935 [8]. Later the tannic acid was found to be active also against other plant viruses [18, 12], and bacteriophages too [6, 7]. In 1948, GREEN [9] reported that tannic acid inhibited the growth of influenza A virus in the allantoic sac, but FRISCH and CARSON [19] then showed that the effect of tannic acid in vivo is transitory as in the 42nd hour of incubation the appearance of influenza virus was demonstrable in the allantoic fluid. These authors supposed that in vivo tannic acid inactivated exclusively the extracellular virions by denaturing their protein shell. CHEO and LINDNER [12] reported that tannic acid inactivated not only the tobacco mosaic virus itself but also its purified RNA component, and they suggested that the inactivating effect of tannic acid might result from its reaction with the protein as well as with the nucleic acid of the virus. COHEN *et al.* [20] demonstrated in aqueous extracts of *Melissa officinalis* an antiviral principle active against Semliki Forest-virus, Newcastle disease virus, Vaccinia virus and *Herpesvirus hominis* grown in embryonated eggs or in chicken fibroblast cells. It was supposed that the active substance may be tannic acid or some other polyphenol derivative. In our studies we succeeded in demonstrating the antiviral effect of commercial tannic acid against *Herpesvirus hominis*, *Herpesvirus suis* and type 3 parainfluenza virus. No effect was demonstrable on poliovirus types 2 and 3 and adenovirus types 3, 4 and 6. Differences were observed in the grade of tannic acid sensitivity of the different viruses. This was shown in the experiments using undiluted virus samples. Under such conditions tannic acid was active against herpesviruses and fully inactive against parainfluenza virus. The difference in tannic acid sensitivity of these two viruses was also demonstrable in respect of the active concentration required for the inactivation of the respective virus.

The action of tannic acid appeared to be virucidal in nature. Tannic acid treatment reduced the titres of certain viruses both *in vivo* and *in vitro*.

Sensitivity of the cells to herpesviruses was also influenced by tannic acid. The results of our experiments did not permit to decide whether cell-bound tannic acid was inhibiting the adsorption of virus or inactivating the adsorbed virions *in situ*.

A virostatic action of tannic acid was not observed. The titres of *Herpesvirus hominis* were even somewhat elevated if tannic acid had been added 2 hours after the adsorption of virus. CHEO and LINDER [12] described the development of a systemic disease in tannic acid treated cucumber plants infected with tobacco mosaic virus which usually causes only local damage. They supposed that tannic acid might react with an interferon-like substance present in the cells, thus interfering with the host's protective mechanism. Our experiments have failed to yield an explanation for the enhancing effect of tannic acid on *Herpesvirus hominis* in a given phase of the viral cycle.

The effect of tannic acid on *Herpesvirus suis* was moderately inhibited by protamine sulphate. Since the latter compound itself has an adsorption inhibiting effect, a more significant action could not be expected. VAHERI [21] showed that protamine sulphate lowered the infectivity of *Herpesvirus hominis*. COLTER *et al.* [22] reported about the inhibitory effect of protamine sulphate in the Mengo encephalomyelitis virus and L-cell system, and presented experimental evidence in favour of the supposition that the primary effect of protamine sulphate consisted of its fixation by the cellular receptors which thus were blocked. A similar explanation might be valid in the *Herpesvirus suis*—chicken fibroblast system studied by us.

Tannic acid is a natural polyanionic compound present in several plants. Polyanions of animal origin are known to inhibit certain viruses. Thus for instance heparin inhibits *Herpesvirus hominis* [23, 24], *Herpesvirus suis*, myxoviruses and West Nile virus [21]. Some synthetic polyanions inhibit *Herpesvirus hominis* as shown by VAHERI [25] as well as TAKEMOTO and FABISCH [26]. The sensitivity of certain viruses and virus mutants to the polyanionic agar inhibitor or dextrane sulphate is well-known [27, 28, 29, 30].

We suppose that the observed antiviral effect of tannic acid is somehow related to the polyanionic nature of the substance. As to its mechanism of action, it may resemble that suggested for the other polyanions and consists of its fixation by the cationic groups of the virion. This reaction probably results in an inactivation of the virus. Differences observable in the sensitivity to tannic acid of different viruses seem to point to the existence of certain differences in the cationic surface groups of the respective virions.

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THE ANTIFUNGAL COMPOUNDS OF CYNANCHUM VINCETOXICUM (L.) PERS

I. THE QUANTITATIVE ANTIFUNGAL SPECTRUM OF SUBSTANCE C-1

By

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Summary. The antifungal activity of the compound isolated from *Cynanchum vincetoxicum* (L.) Pers and termed Substance C-1 has been studied against 40 yeasts, 40 moulds and 20 dermatophytes. With the exception of some of the moulds most of the examined species and strains including all of the human pathogenic fungi tested were found to be highly sensitive. The minimum inhibitory concentration on glucose-bouillon-peptone culture medium at pH 7 is between 0.015 and 1 μg/ml.

On examining more than 2500 plant species [FERENCZY and ZSOLT, 1] for new sources of antibiotics against bacteria *(Staphylococcus aureus* Walker, *Bacillus cereus var. mycoides*, *Escherichia coli O* 111, *Serratia marcescens)* and fungi *(Procandida albicans*, *Hansenula anomala*, *Aspergillus niger*, *Syncephalastrum racemosum)* it was established that certain preparations from *Cynanchum vincetoxicum* exert a significant antifungal effect but do not inhibit growth of the bacteria tested. In contrast to compounds of identical selectivity occurring in other plants, these Cynanchum preparations showed no haemolytic effect. As a decrease of the pH of the culture medium involved a significant decrease in antifungal effect, it seemed likely that a compound of basic character was responsible for the inhibition. Thin-layer chromatography revealed that the antifungal effect was due to not one but three compounds [2].

Independently of the above mentioned examinations, HÁZNAGY, TÓTH and SZENDREI [3] have succeeded in isolating a substance from the roots of *Cynanchum vincetoxicum*. Qualitative tests proved this substance to be of alkaloid character, with a melting point of 212—214° C, giving Dragendorf-positive reaction on the thin-layer chromatogram and a positive qualitative N-test. The compound forms adducts with sulphonic acid dyes (bromthymol blue, bromcresol green) well soluble in organic solvents.

Comparative thin-layer chromatography revealed that the isolated substance brings about a zone of fungal inhibition at the same R_f-value as the most apolar antifungal compound of the plant. This compound has been termed Substance C-1.

Materials and methods

The fungi used in the experiments originated from collections of the Institute of Plant Physiology, Attila József University, Szeged, State Institute of Hygiene, Budapest and from the Ampelological Research Institute, Budapest.

Substance C-1 was dissolved in 0.01 *N* HCl, mixed with an adequate quantity of culture medium to obtain two-fold dilution series in the range of 2—0.004 μg/ml.

The medium consisted of beef-extract, 6 g; peptone, 4 g; NaCl, 2 g; glucose, 10 g; agar, 20 g; and distilled water up to 1000 ml and was adjusted with Na_2CO_3 to pH 7.

To secure the most possible uniformity of conditions, the large plate technique [4] was used. The medium was 3 mm thick. After solidification the surface was inoculated as follows.

Yeasts. A suspension of 10 ± 5 million cells per ml was made from 24-hour malt agar cultures of the strains. From the suspensions a loopful (about 0.001 ml) was applied to a spot of 5 mm in diameter.

Moulds. Conidia or spores were inoculated onto the culture medium in Petri dishes. After 48-hour incubation 2.5 mm wide columns were cut off and lifted from the outermost part of the colonies by means of a special borer, and so transferred onto the test medium containing the active agent as to ensure the mycelium's close connection with the medium.

Dermatophytes. Mats 3 mm in diameter from a two-week old culture grown on Sabouraud's medium was placed on the test media containing the active compound.

The inoculated media were incubated at 30°C. The results in the case of yeasts and moulds were read after 48 hours, while in the case of dermatophytes, after 2 weeks. In the yeasts every case was considered to be positive when growth was noted even if it was sporadic and hardly discernible. In moulds each case was considered positive where hyphae appeared around the column, and with dermatophytes when mycelium began to form on the surface of the culture medium.

The results have been arranged on the basis of 3 parallel examinations. Whenever growth could be detected, it was indicated as positive in the Tables.

Results and discussion

Results are shown in Tables I, II, and III. "+" means growth and "—" lack of growth.

The examination has been extended to 33 strains of *Procandida (Candida) albicans* recently isolated from patients at the Mycological Laboratory of the State Institute of Hygiene in order to determine the range of variation in sensitivity. Results are assembled in Table IV.

In yeasts and still more in filamentous fungi a partial growth inhibition was shown in a fairly wide concentration range. In Fig. 1 can be seen the inhibiting effect on the growth of some species of moulds compared with the control. The data show results of 10 parallel examinations read in the 48th hour of incubation. Maximal standard error was ± 1.8 per cent.

Cynanchum vincetoxicum have played a significant role in therapy in bygone days, as shown by the name "vincetoxicum" and also some old literary data [6, 7, 8].

Of the antifungal substances isolated from the plant, Substance C-1 is an antifungal compound of broad spectrum and high activity. At low concentration of 0.015 to 1 μg/ml it fully inhibited growth of nearly all the species and strains examined. Partial inhibition was observed even at a concentration of 0.008 μg/ml.

Whereas some *Aspergillus*, *Penicillium* species and *Rhizopus arhizus* proved resistant, other species from the same families were considerably sensitive. The minimum inhibitory concentration was 0.03 to 0.25 μg/ml for all the

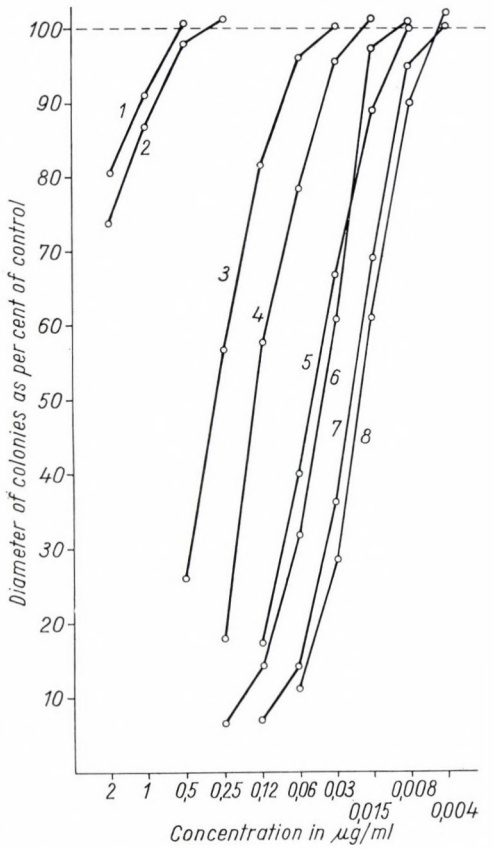

Fig. 1. Effect of Substance C-1 on growth rate of colonies of some mould species. 1 : *Aspergillus niger*, 2 : *Aspergillus phoenicis*, 3 : *Mucor racemosus*, 4 : *Mucor saturninus*, 5 : *Aspergillus ochraceus*, 6 : *Rhizopus nigricans*, 7 : *Aspergillus oryzae*, 8 : *Actinomucor repens*

examined strains of *Procandida (Candida) albicans*, and 0.03 μg/ml for *Cryptococcus neoformans*. Dermatophytes were without exception sensitive to 0.03 to 1 μg/ml.

Acknowledgements. We are indebted to *Dr.* E. K. Novák (Department of Mycology, State Institute of Hygiene) and to Mrs. E. Nyerges (Ampelological Research Intitute) for a collection of fungi, and to Miss T. Bognár for skilled technical assistance.

Table I

Effect of Substance C-1 on yeasts
(System of yeasts by NOVÁK and ZSOLT [5])

Species	Concentration, μg/ml								
	1	0.5	0.25	0.12	0.06	0.03	0.015	0.008	0.004
Lipomycetaceae									
Nadsonia slovaca	−	+	+	+	+	+	+	+	+
Saccharomycetaceae									
Saccharomyces terricolus	−	−	−	−	−	−	−	+	+
,, *rouxii*	−	−	−	−	−	+	+	+	+
,, *oviformis* strain Xeres	−	−	−	−	+	+	+	+	+
,, *cerevisiae* strain Rasse XII	−	−	−	−	−	+	+	+	+
,, ,, strain Mauthner	−	−	−	−	−	+	+	+	+
,, ,, strain Tokaj 22	−	−	−	−	−	+	+	+	+
,, ,, strain Kecskemét 5	−	−	−	−	−	+	+	+	+
,, *pretoriensis*	−	−	−	+	+	+	+	+	+
,, *carlsbergensis*	−	−	−	−	−	+	+	+	+
,, *diastaticus*	−	−	−	−	−	−	+	+	+
Zymodebaryomyces globosus	−	−	+	+	+	+	+	+	+
Hansenulaceae									
Endomycopsis fibuliger	−	−	−	−	−	+	+	+	+
Zymopichia pijperi	−	−	−	−	+	+	+	+	+
,, *saitoi*	−	−	−	−	−	−	+	+	+
Hansenula anomala	−	−	−	−	+	+	+	+	+
Fabosporaceae									
Kluyveromyces polysporus	−	−	+	+	+	+	+	+	+
Dekkeromyces delphensis	−	−	+	+	+	+	+	+	+
,, *fragilis*	−	−	−	−	+	+	+	+	+
Sporobolomycetaceae									
Sporobolomyces pararoseus	−	−	−	−	+	+	+	+	+
Cryptococcaceae									
Geotrichum linkii	−	−	+	+	+	+	+	+	+
,, *candidum*	−	−	+	+	+	+	+	+	+
,, *matalense*	−	−	+	+	+	+	+	+	+
Procandida stellatoidea	−	−	−	−	+	+	+	+	+
,, *albicans* strain CBS	−	−	−	+	+	+	+	+	+
Azymocandida aaseri	−	−	−	−	−	+	+	+	+
,, *corniculata*	−	−	−	−	+	+	+	+	+

(Table I continued)

Species	Concentration, μg/ml								
	1	0,5	0.25	0.12	0.06	0.03	0.015	0.008	0.004
Candida krusei	—	—	—	+	+	+	+	+	+
,, *famata*	—	—	—	+	+	+	+	+	+
,, *parapsilosis*	—	—	—	+	+	+	+	+	+
,, *pulcherrima*	—	—	—	+	+	+	+	+	+
,, *utilis*	—	—	—	—	—	—	+	+	+
,, *guilliermondii*	—	—	—	+	+	+	+	+	+
Torulopsis vanzylii	—	—	—	—	+	+	+	+	+
,, *stellata*	—	—	—	+	+	+	+	+	+
,, *inconspicua*	—	—	—	—	+	+	+	+	+
Paratorulopsis pseudaeria	—	—	—	—	+	+	+	+	+
Cryptococcus neoformans	—	—	—	—	—	—	+	+	+
,, *diffluens*	—	—	—	+	+	+	+	+	+
Nigrococcus nigricans	—	—	—	+	+	+	+	+	+
Rhodotorula rubra	—	—	+	+	+	+	+	+	+

Table II

Effect of Substance C-1 *on moulds*

Species	Concentration, μg/ml								
	1	0.5	0.25	0.12	0.06	0.03	0.015	0.008	0.004
Mucorales									
Absidia orchidis	—	—	+	+	+	+	+	+	+
Actinomucor repens	—	—	—	—	+	+	+	+	+
Circinella minor	—	—	+	+	+	+	+	+	+
Cunninghamella echinula	—	—	—	—	+	+	+	+	+
Mucor fragilis	—	—	+	+	+	+	+	+	+
,, *hiemalis*	—	+	+	+	+	+	+	+	+
,, *racemosus*	—	+	+	+	+	+	+	+	+
,, *ramannianus*	—	—	+	+	+	+	+	+	+
,, *saturninus*	—	—	+	+	+	+	+	+	+
Rhizopus arhizus	+	+	+	+	+	+	+	+	+
,, *circinans*	—	—	+	+	+	+	+	+	+
,, *nigricans*	—	—	+	+	+	+	+	+	+
Syncephalastrum racemosum	—	—	—	+	+	+	+	+	+

(Table II continued)

Species	Concentration, μg/ml								
	1	0,5	0,25	0,12	0,06	0,03	0,015	0,008	0.004
Fungi imperfecti									
Aspergillus awamori	+	+	+	+	+	+	+	+	+
,, *elegans*	+	+	+	+	+	+	+	+	+
,, *flavus*	−	−	−	+	+	+	+	+	+
,, *foetidus*	+	+	+	+	+	+	+	+	+
,, *fumigatus*	−	−	−	+	+	+	+	+	+
,, *japonicus*	+	+	+	+	+	+	+	+	+
,, *nidulans*	−	+	+	+	+	+	+	+	+
,, *niger*	+	+	+	+	+	+	+	+	+
,, *ochraceus*	−	−	−	+	+	+	+	+	+
,, *oryzae*	−	−	−	+	+	+	+	+	+
,, *phoenicis*	+	+	+	+	+	+	+	+	+
,, *proliferans*	+	+	+	+	+	+	+	+	+
,, *repens*	−	−	+	+	+	+	+	+	+
,, *wentii*	−	−	−	+	+	+	+	+	+
,, *terrens*	+	+	+	+	+	+	+	+	+
Penicillium candido-fulvum	+	+	+	+	+	+	+	+	+
,, *digitatum*	−	−	+	+	+	+	+	+	+
,, *fasciculatum*	+	+	+	+	+	+	+	+	+
,, *frequentans*	+	+	+	+	+	+	+	+	+
,, *javanicum*	−	−	−	−	+	+	+	+	+
,, *lanosum*	+	+	+	+	+	+	+	+	+
,, *levitum*	−	−	−	−	+	+	+	+	+
,, *martensii*	+	+	+	+	+	+	+	+	+
Scopulariopsis brevicaulis	−	+	+	+	+	+	+	+	+
Botrytis cinerea	−	−	−	−	+	+	+	+	+
Gibberella fujikuroi	−	−	−	+	+	+	+	+	+
Trichoderma glauca	−	−	−	+	+	+	+	+	+

Table III

Effect of Substance C-1 on dermatophytes

Species	Concentration, μg/ml								
	1	0.5	0.25	0.12	0.06	0.03	0.015	0.008	0.004
Achorion ferrugineum	—	—	—	—	—	+	+	+	+
,, *schoenleinii*	—	—	—	—	—	+	+	+	+
Epidermophyton folccosum	—	—	+	+	+	+	+	+	+
Kaufmannwolfia pedis	—	—	—	—	+	+	+	+	+
Keratinomyces ajelloi	—	—	—	—	+	+	+	+	+
,, *longifusus*	—	—	—	+	+	+	+	+	+
Microsporon audouini	—	—	—	—	—	+	+	+	+
,, *canis*	—	—	—	—	+	+	+	+	+
,, *cookei*	—	—	—	—	—	—	+	+	+
,, *fulvum*	—	—	—	+	+	+	+	+	+
,, *gypseum*	—	—	—	+	+	+	+	+	+
,, *nanum*	—	—	—	+	+	+	+	+	+
,, *vanbreuseghemi*	—	+	+	+	+	+	+	+	+
Trichophyton gallinae	—	—	+	+	+	+	+	+	+
,, *megninii*	—	—	—	—	—	+	+	+	+
,, *mentagrophytes*	—	—	—	—	—	+	+	+	+
,, *soudanense*	—	—	—	—	+	+	+	+	+
,, *terrestre*	—	—	—	—	+	+	+	+	+
,, *verrucosum*	—	—	—	—	—	—	+	+	+
,, *violaceum*	—	—	—	—	—	—	+	+	+

Table IV

Effect of Substance C-1 on Procandida (Candida) albicans strains of human origin

Strain	Concentration µg/ml				
	0.25	0.12	0.06	0.03	0.015
9/ 89	—	—	—	+	+
9/121	—	—	—	—	+
9/122	—	—	—	+	+
9/132	—	—	—	+	+
9/134	—	—	—	+	+
9/160	—	—	+	+	+
9/162	—	—	—	+	+
9/174	—	+	+	+	+
9/178	—	+	+	+	+
9/204	—	—	—	+	+
9/205	—	—	—	+	+
9/207	—	—	—	+	+
9/221	—	—	—	+	+
9/233	—	—	—	+	+
9/237	—	—	—	+	+
9/238	—	—	—	+	+
9/242	—	—	—	—	+

Strain	Concentration, µg/ml				
	0.25	0.12	0.06	0.03	0.015
9/245	—	—	—	+	+
9/246	—	—	—	+	+
9/247	—	—	+	+	+
9/253	—	—	+	+	+
9/259	—	—	—	—	+
9/269	—	—	—	+	+
9/270	—	—	—	+	+
9/278	—	—	—	+	+
9/293	—	—	—	+	+
9/303	—	—	—	+	+
9/306	—	—	—	+	+
9/307	—	—	—	+	+
9/316	—	—	+	+	+
9/318	—	—	—	+	+
9/319	—	—	+	+	+
9/320	—	—	—	+	+

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STUDIES ON THE CONDITIONS OF INTERFERON PRODUCTION BY CELLS INFECTED WITH HERPESVIRUSES

By

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Summary. The production of a virus growth inhibitory principle has been examined in secondary human embryonic fibroblast cell cultures infected with *Herpesvirus hominis*, *Herpesvirus varicellae* and Cytomegalovirus. All the three viruses were found to induce the production of a virus inhibitory substance. The amount of this was the greatest in cultures infected with Varicella virus, less in those supporting the growth of Cytomegalovirus and the least in cells infected with Herpes simplex virus. The virus inhibitory substance was titrated in secondary human embryonic fibroblast, HeLa and III/1 permanent monkey kidney cells. The highest titres were obtained in the last group. The inhibitory substance is either interferon or an interferon-like principle. The relation between the amount of inhibitory substance produced and the progress of viral infection of the tissue culture is discussed.

Since the discovery of interferon by Isaacs and Lindenmann [1], many workers have studied its production in a number of viral infections. It was found that interferon production was induced by a variety of viruses [2—11, 13—18, 22] and even by infection with purified viral nucleic acid [19]. Bacterial infections have also been shown to initiate the formation of interferon [26]. The interferon production inducing capacity of the human pathogenic group of Herpesviruses is a less extensively studied field. These viruses being known to cause latent infections it seemed interesting to examine whether this character has any relation to their capacity to induce interferon production.

Materials and methods

Virus strains. Herpes simplex virus. The strain isolated in this laboratory [25] and maintained by serial transfers in HeLa cells was used. *Herpesvirus varicellae* was isolated from the contents of a vesicle and maintained in human embryonic fibroblast cell cultures. *Cytomegalovirus.* The strain was kindly supplied by *Dr.* H. Stern (St. George's Hospital Medical School, University of London) and maintained in cultures of human embryonic fibroblasts.

Tissue culture. If not stated otherwise, human embryonic fibroblast (HEF) cell cultures were used as described earlier [24].

For the production of interferon secondary cultures of HEF were used in 1 litre Roux flasks. Interferon titrations were performed in tube cultures of HEF, III/1 permanent monkey kidney (PMK) [23] or HeLa cell cultures.

Media. Tissues used for interferon production were fed with a mixture of 50 per cent each of Parker's 199 and Hanks' media containing a final concentration of 2 per cent calf serum. The same medium was used for titrations but the calf serum content was elevated to 5 per cent.

Production of interferon. HEF cultures in one litre Roux flasks were infected with one of the three Herpesviruses in 5 to 6 $\times$ 10^3 CPD_{50} individual doses. Infected cultures were incubated at 37° C and examined daily under the microscope. The fluid phase was harvested as soon

as the cytopathic effect (CP) became markedly positive (+++). This was achieved after 4, 6 to 8, and 10 days with Herpes simplex, Cytomegalo- and Varicella-viruses, respectively. After centrifugation of the harvested fluid, it was kept at +4° C for 24 hours, and stored at −20° C until used. Infected and control cultures were treated further as follows. Thawed material was transferred to dialysis bags and evaporated by ventilation in the cold room (+4° C). When about a 20fold concentration was achieved the pH was adjusted to 2.0 by adding 5 M hydrochloric acid. The acidified material was allowed to stand at +4° C for 24 hours. The pH was then readjusted to 7.2 by adding 5 M sodium hydroxide and dialysed against lactalbumin hydrolysate and indicator-free Hanks' solution for 4 days. The material thus obtained was tested for sterility and virus inhibitory activity. The absence of infective viruses was checked by inoculation of HEF cell cultures.

Infectivity titrations were carried out in HEF cell cultures using the fluid phase of cultures infected with Herpes simplex or Cytomegalovirus and the infected cells themselves from cultures inoculated with Herpesvirus varicellae. Titrations were performed in tube cultures by inoculating 0.1 ml samples of each dilution step to each of 4 tubes. Infective titres were calculated by the Kärber method.

Titration of interferon. Interferon preparations obtained as described were so diluted as to represent a fivefold or identical concentration as referred to the original starting material. One ml of each was used for preincubation of HEF, HeLa and III/1 PMK cells for 24 hours at 37° C. The cultures were then infected with 100 plaque forming units of vaccinia virus and incubated for 44 hours at 37° C. The plaques formed were counted after staining with 0.1 per cent aqueous fuchsin solution. The inhibitory effect was evaluated as reduction of the plaque count referred to the control. Figures given in the Table are mean values of 3 parallel experiments. In every experiment, 4 parallel tubes were used per dilution.

Growth curves. Each of four tubes with HEF cell cultures were inoculated with 0.1 ml samples of a tenfold dilution series of Herpes simplex, Varicella or Cytomegaloviruses. The progress of CP effect was examined daily and the CPD_{50} values were calculated. Growth curves were constructed by plotting the daily CPD_{50} values against time.

Results and discussion

In cultures of human embryonic fibroblast cells the growth of Herpes simplex virus was good, while that of Cytomegalovirus and Varicella virus was relatively slow, as estimated on the basis of the appearance of the cytopathogenic effects. Results are presented in Fig. 1.

It has long been known that in infected cultures Herpes simplex virus is present in large amounts in the extracellular fluid. The amount of extracellular virus in Cytomegalovirus infected cultures is relatively low, while Varicella

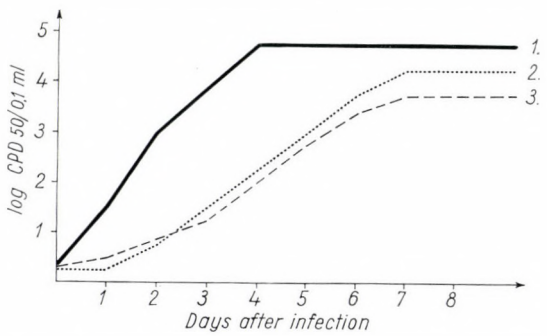

Fig. 1. Titres of Herpes simplex, *Herpesvirus varicellae* and Cytomegalovirus in human fibroblast cultures after inoculation

1 : Herpes simplex virus; 2 : Cytomegalovirus; 3 : Varicella virus

virus is present practically exclusively in the intracellular form. Thus the three viruses used in our experiments differed not only in their growth rates but also in the completeness of their maturation process. It seemed therefore of interest to examine the possible presence of an interferon-like substance in cultures which had been infected with one of the above viruses and reached a given phase of virus maturation. For this purpose, tissue cultures prepared in Roux flasks were infected as described above. When the infected cultures exhibited a (+++) grade of degeneration, the concentration of growth inhibitory substance in the medium was determined. These determinations were performed on human embryonic fibroblast cultures, HeLa cells and III/1 permanent monkey kidney cells, each infected with equal doses of vaccinia virus. Results are presented in Table I.

Table I

Inhibition of the PFU production of vaccinia virus in per cents, in the presence of interferon induced by different viruses

Cell culture	Interferon produced by					
	Herpesvirus varicellae		Cytomegalovirus		Herpes simplex virus	
	Original	5× conc.	Original	5× conc.	Original	5× conc.
Human fibroblast	40	60	25	48	20	20
HeLa	40	95	<10	48	<10	<10
III/1 monkey kidney	85	90	40	50	42	46

As shown by the data presented in Table I, a substance capable of inhibiting the growth of vaccinia virus was detectable in the fluid of cultures infected with any of the three viruses used. The highest amounts of inhibitory substance were produced parallel to the replication of Varicella virus. Production was lower in Cytomegalovirus infected cultures and lowest in those supporting the growth of Herpes simplex virus. The fluid phases of the latter cultures contained very little inhibitory substance even after fivefold concentration. The amount of inhibitory substance produced as a result of Varicella virus replication was about 3 times more than that produced by Herpes simplex virus. The inhibitory activity of nutrient fluid of Cytomegalovirus infected cultures was intermediate. Permanent monkey kidney cells III/1 were found to be the most sensitive type of cells for demonstration of the inhibitory substance.

As to the nature of the inhibitory substance, it proved to be a non-dialysable, acid resistant proteinaceous material stable for several months at +4° C. Precipitated by acetone according to ZEMLA and VILČEK [22], the substance could be redissolved in a fully active form. The substance was active against

viruses other than vaccinia, too. Taking all this into account, we suppose the material to be interferon-like.

The amount of infectious virus in cultures used for interferon production was determined as described under "Materials and methods". In cultures infected with Herpes simplex virus, large amounts of extracellular virus were detectable. The amount of virus in the fluid was less in Cytomegalovirus infected cultures, while no extracellular virus was detectable in Varicella virus infected cultures.

As to the interferon-producing capacity of human pathogenic members of the Herpesvirus group, we are only aware of one study performed with Herpes simplex virus *viz.* that of FRUITSTONE *et al.* [7] who demonstrated the appearance of interferon in the allantoic fluid of Herpesvirus infected embryonated eggs.

In tissues infected with Herpes simplex virus, the production of interferon may be of great importance in the development of latent infections and persistence. This view has been supported by the studies of GLASGOW and HABEL [12] in tissue cultures persistently infected with polyoma and Herpes simplex virus. Using III/1 permanent monkey kidney cells, GÉDER *et al.* [20] produced persistent infection with Herpes simplex and Varicella virus. In preliminary experiments the concentrated tissue culture fluid of the Varicella-virus carrying III/1 cell line had an inhibitory effect on the PFU of vaccinia virus. According to WADDEL *et al.* [10], the development of a persistent Herpes simplex virus infection in tissue cultures in the absence of antibodies should be ascribed to the production of an interferon-like substance.

According to our own results, also other human pathogenic members of the Herpesvirus group (Cytomegalo and Varicella-zooster) produce a virus growth inhibitory substance during their replication.

Since the extent of interferon production and the appearance of free virus are inversely related, *viz.* with Herpes simplex large amounts of virus are liberated and little interferon is produced, with Varicella virus the case is the opposite and Cytomegalovirus is in both respects intermediate between the two, the above phenomena might indicate the differences encountered in the multiplication ability and maturation process of the three kinds of viruses. These characteristics might be of great importance in the pathomechanism of the diseases produced by viruses belonging to this group and in the development of latent infections characteristic of them.

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ANIMAL EXPERIMENTS CONCERNING THE AETIOLOGY OF VIRAL HEPATITIS

By

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Summary. Attempts have been made to reproduce the experiments of BERTÓK *et al.*, *i.e.*, to render rats susceptible to human viral hepatitis by adding 0.3 per cent ethionine (daily dose, 23—25 mg) to a methionine-deficient diet. Rats kept on this diet (Group I) as well as those kept on normal diet (Group III) were treated with material obtained from patients suffering from acute hepatitis. The animals and adequate noninfected controls (Groups II and IV) were kept under observation to record jaundice and death. Serum samples were tested for haemagglutination, and transmission of the haemagglutinating factor to embryonated eggs was attempted. The serum proteins of the animals were analyzed electrophoretically. There were significant differences between Group I and the other groups in the relative level of gamma globulin, in the haemagglutinating capacity of the serum and in the results of egg inoculations. Since, on the other hand, jaundice and even deaths occurred in the ethionine-fed "noninfected" group (Group II) too, and the haemagglutinating agent was recovered from the liver extract of two "infected" animals kept on the normal diet (Group III) conclusions of BERTÓK *et al.* could be neither confirmed nor refuted.

Investigation into the aetiology of human viral hepatitis is greatly inhibited by the lack of a susceptible experimental host. BERTÓK [1, 2] attempted to overcome the "indifference" of experimental animals to the hepatitis agent by keeping them on a methionine-deficient diet to which, as methionine-antagonist, ethionine was added. The animals thus treated have been reported to react to human hepatitis virus with an illness resembling human hepatitis in clinical symptoms and histological lesions, most of the animals even died.

We have made an attempt at reproducing these experiments. The histological examinations were carried out in the First Institute of Pathology and Experimental Cancer Research, University Medical School, Budapest. The results of these studies are published in an accompanying report. In the present paper some additional laboratory investigations will be discussed.

Materials and methods

Experimental animals. Leptospira- and Bartonella-free albino rats of 200 g average body weight were used. Guinea pigs were also kept on the ethionine diet, but most of these animals died without further treatment. The rats were divided into four groups. The experimental group (Group I) was kept on the ethionine diet and inoculated with hepatitis material. Among three control groups, Group II was kept on the ethionine diet but was not inoculated, Group III was kept on the normal diet and inoculated with hepatitis material, whereas Group IV was the normal control group.

Diet. As a normal diet, the one recommended by Sós and Lődy [1, 3] was given. Composition of this diet was 60 per cent starch, 5 per cent sugar, 20 per cent casein, 1 per cent brewer's yeast, 4 per cent salt mixture, 10 per cent fat mixture, 0.1 per cent vitamin mixture.

Ethionine diet: 62 per cent starch, 4.6 per cent sugar, 8 per cent casein, 8 per cent size, 3 per cent brewer's yeast, 4 per cent salt mixture, 10 per cent fat mixture, 0.1 per cent vitamin mixture, 0.3 per cent ethionine.

Composition of mixtures. Salt mixture: 63.44 g NaCl, 102 g KCl, 342 g KH_2PO_4, 170 g $MgSO_4 \cdot 7H_2O$, 0.24 g $CuSO_4 \cdot 5H_2O$, 1.85 g $MnCl_2 \cdot 4H_2O$, 0.02 g KI, 1.5 g $ZnCl_2 \cdot H_2O$, 0.05 g As_2O_3, 314 g Ca lactate, 4.9 g Fe(III) citrate for every 1000 g. Vitamin mixture: 2.0 mg aneurin-HCl, 3.0 mg riboflavin, 2.0 mg pyridoxine, 15 mg pantothenic acid, 15 mg niacin-amide, 5.0 mg p-amino-benzoic acid, 1.0 mg folic acid, 0.1 mg biotin, 50 mg *a*-tocopherol, 4.0 mg vitamin K, 1500 U calciferol, and starch to 1000 mg. Fat mixture: equal parts of tallow, olive oil and margarine.

The rats kept on the ethionine diet consumed a daily dose of 23—25 mg of ethionine over 52 days.

Hepatitis materials. Serum and faecal samples were collected from patients as early as possible after the acute disease had been diagnosed, *i.e.*, within 24 hours after admission into hospital. Faecal suspensions obtained from four patients were mixed and filtered through Seitz EK pad. When the inoculum was serum, this was a mixture of samples taken from two patients. The sera were stored in the frozen state (about —4° C) for a week and heated to 60° C for 30 minutes before used, whereas the faecal filtrates were inoculated immediately, without any pretreatment. In the present experiments two faecal and two serum mixtures were used. The former were administered orally, twice 0.2 ml per animal, the latter parenterally, twice 0.1 ml per animal. Further animals were inoculated with 0.1 ml of allantoic fluid from the 11th passage of a haemagglutinating agent originally obtained by allantoic inoculation of acute-phase hepatitis serum.

Blood samples. Blood was taken from the tail vein on the 15th and 22nd days after the first inoculation with hepatitis material. One rat in each group was exsanguinated on the 24th day, the survivors on the 40th day.

Analysis of serum proteins. Serum proteins were analyzed by horizontal, low-voltage paper electrophoresis [4].

Haemagglutination (HA). Human Rh-positive, group O erythrocytes were treated with 0.5 per cent tannin. We have shown [5] that acute-phase serum samples of hepatitis patients agglutinate tannin-treated erythrocytes. The reaction appeared to be specific for viral hepatitis.

Inoculation of embryonated eggs. In the course of our earlier studies [6], 11-day preincubated hen's eggs were inoculated with acute-phase hepatitis sera into the allantoic cavity. After three days reincubation the allantoic fluid of some inoculated eggs contained a haemagglutinating factor which seemed to be identical with the serum factor. The haemagglutinating factor was maintained over 20 consecutive passages in embryonated eggs. The egg-passaged haemagglutinin agreed with the serum factor (i) in being extraordinarily resistant to heat; (ii) in its haemagglutination spectrum and (iii) in being neutralizable both with convalescent-phase sera of patients suffering from hepatitis and with the sera of rabbits immunized with haemagglutinating allantoic fluid. All serum samples were heated to 60° C for 30 minutes before inoculation into eggs. The passaged allantoic fluids were subjected either to the same procedure or were kept in a boiling water bath for 15 minutes. Such heat treatment failed to influence the HA titres. Detailed virological, biochemical and physicochemical examination of the haemagglutinating agent is in progress.

In the course of the present experiments serum samples, liver homogenates, and liver extracts prepared by soaking livers in saline were inoculated into the allantoic cavity of embryonated eggs. The harvested allantoic fluids were kept at 100° C for 15 minutes and then titrated with tannin-treated human Rh-positive group O erythrocytes.

Anti-HA sera. Rabbits were immunized with haemagglutinating allantoic fluid kept at 100° C for 15 minutes before use. The schedule of immunization was that recommended by Gell and Coombs [7]. On the first day 1 ml was injected into each of the four thighs, on the 15th day 1 ml into each of the four shoulders, intramuscularly. From the 21st to the 33rd day increasing doses (from 0.2 ml to 1.6 ml) were injected intravenously every second day. The rabbits were exsanguinated on the 10th day after the last injection. To absorb the antibodies produced against the normal proteins of the allantoic fluid, the sera were mixed with equal volumes of normal allantoic fluid and incubated at 37° C for 60 minutes and, subsequently, at room temperature for 60 minutes. The HA-inhibiting (HI) titres of the sera thus obtained wer: (a) 1: 32, (b) 1 : 32 and (c) 1 : 64 when titrated against 12 HA doses in Takátsy's Microtitrator [8].

Results

In the main experiment, observations were extended to (i) death, (ii) jaundice, (iii) serum gamma globulin in per cent of total serum protein and (iv) serum haemagglutinins to tanninized human erythrocytes.

Nearly half of the ethionine control group (Group II), more than half of the orally infected group (Group I/a), and nearly three-quarters of the parenter-

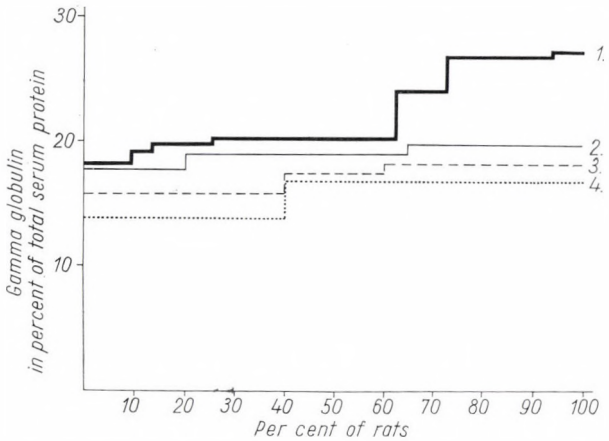

Fig. 1. Relative serum gamma-globulin level in groups I—IV
1 = Ethionine-fed, hepatitis infected; 2 = Ethionine-fed, noninfected; 3 = Normal diet, hepatitis infected; 4 = Normal diet, noninfected

ally inoculated group (Group I/b) died in 40 days after the first treatment with hepatitis material.

Jaundice occurred in each of the ethionine-fed groups, *viz.* in three animals in the ethionine control group, somewhat more frequently in the orally infected group, and in approximately three-quarters of the animals infected parenterally.

There was a marked rise in relative serum gamma-globulin level in the ethionine-treated animals. The rise was more pronounced in the infected than in the control groups. If, arbitrarily, 20 per cent is accepted as the upper limit of normal, the difference between the experimental group (Group I) (>20 per cent of 31 animals out of 76) and the control groups (1 animal out of 40) is significant statistically. (It should be noted that the total serum protein level was not determined.)

Agglutination of tanninized erythrocytes occurred only with serum samples taken from the experimental group (Group I). Inoculation of embryonated eggs was also more successful in this group. In 19 cases, allantoic fluid inoculated with serum samples gave a positive HA, whereas in 7 cases inocula-

Table I

Experiments with

Group	Diet	Inoculum	Route of inoculation	No. of rats	Rats died	
					No.	%
I/a	Ethionine	Faeces	oral	20	12	60
I/b	Ethionine	Serum or allantoic fluid	parenteral	56	44	79
II	Ethionine	—	—	20	9	45
III	Normal	Faeces	oral	10	—	—
IV	Normal	—	—	10	—	—

* Not neutralizable with anti-HA immune serum

tion was successful with liver extracts (26 positive results from 76 rats). In the control groups, inoculation of eggs was positive in three cases out of the 30; in two cases eggs inoculated with liver extract of infected rats kept on normal diet (Group III) showed HA (one of the positive rats was exsanguinated on the 24th day after infection), the third positive animal was a non-infected rat which had been kept on the ethionine diet (Group II). In this case the positive eggs had been inoculated with liver homogenate. This haemagglutinating factor was, however, different from the others in that it was less thermostable (it was inactivated at 100° C within 10 minutes) and neither inhibited nor neutralized by anti-HA rabbit sera. All the haemagglutinating allantoic fluids proved to be sterile bacteriologically.

In addition to the experiments summarized in Table I, an experiment was initiated, which was, however, disturbed unexpectedly. A bacteriologically sterile allantoic fluid of the 11th passage of a haemagglutinating agent was boiled for 15 minutes, and 0.1 ml of the fluid was injected parenterally into each of 60 rats which had received for 10 days an alternating diet, *viz.*, every other day the normal, and on the other days the ethionine diet. On the 8th, 9th and 10th days after infection, 25 rats (42 per cent) died; 9 of these displayed pronounced jaundice. At necropsy all the animals but six yielded no considerable gross lesions including those characteristic of ethionine. The liver of six rats was smaller than usual and showed petechiae. The unexpected death of animals prevented us from carrying out other investigations.

The survivors, distributed in four groups were kept on the normal diet, Group I was treated with a total dose of 0.75 ml per animal of anti-HA rabbit serum, Group II with 0.5 ml per animal of human gamma globulin ("Human" Institute for Serobacteriological Production and Research, Budapest); and Group III with penicillin and streptomycin. Group IV was not treated.

ethionine-fed rats

Jaundice		Serum haemagglutinin		Successful transmission into eggs		Serum gamma globulin >20%	
No.	%	No.	%	No.	%	No.	%
5	25	5	25	8	40	6	30
41	73	26	46	18	31	25	45
3	15	—	—	1*	5	1	5
—	—	—	—	2	20	—	—
—	—	—	—	—	—	—	—

The experiments were brought to an end when all animals in Group IV had died on the 22nd day after infection. At that time 7 of the 9 rats of Group I, and 5 of the 10 rats of Group II were still living, whereas all the antibiotic-treated animals had died.

Table II

Transmission of haemagglutinating agent into embryonated eggs

Group	No. of rats	Successful transmission with				Successful transmission total
		incubation serum	serum on the 40th day after infection	liver extract	liver homogenate	
I/a	20	1	n.t.	7	—	8
I/b	56	1	17	n.t.	n.t.	18
III	10	—	—	2	—	2

Discussion

The present studies were aimed at reproduction of the experiments of BERTÓK *et al.* concerning the transmission of human viral hepatitis to ethionine-treated rats. The most important proof adduced by these authors was the histological finding.

Among the criteria studied by us we consider the transmission of the factor agglutinating human Rh-positive, Group O erythrocytes to be the most specific for viral hepatitis. This factor was absent from the serum of all the 30 control animals, but was demonstrable in 41 per cent of the ethionine-treated rats inoculated with hepatitis material. With the serum of these animals, but never with the control serum samples, the factor proved to be transmissible to embryonated eggs. However, with the liver extract the agent was trans-

missible to embryonated eggs not only from these animals, but even from two apparently seronegative "infected" animals that had been kept on a normal diet.

Jaundice and death occurred in all the ethionine-treated groups, at the highest rate in Group I, that was infected with hepatitis material.

From the paper-electrophoretic analysis of the serum proteins, the differences in the relative gamma-globulin level might be emphasized, which was more or less increased in each of the groups treated with ethionine and/or hepatitis material (Groups I—III) as compared to the normal control group (Group IV). Considering the different treatment of the groups, the increase in the gamma-globulin level cannot be attributed to a common factor. Instead, according to DITTMER's pathogenetic classification [4], the presence of different types of secondary dysproteinaemia might be supposed. In the infected rats kept on the normal diet (Group III) the statistically nonsignificant increase of the relative gamma-globulin level might be attributed to an activation of the RES, resulting in an immune response to any infective agent introduced orally with the hepatitis material. The more significant dysproteinaemia of the ethionine-fed, noninfected Group II might have been due to the hepatic and pancreatic injuries induced by ethionine. The still higher relative level of gamma globulin in the ethionine-fed infected groups suggests that on the effect of the ethionine diet some injury was added to that caused by the hepatitis material.

Some similar conclusion might be drawn from the experiment which was interrupted by the death of the animals. The unexpected course of this experiment prevented us from studying events more thoroughly. The experiment will be repeated under well-controllable circumstances.

On the basis of the above observations we can neither confirm nor refute the conclusions of BERTÓK *et al.*

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HISTOPATHOLOGICAL FINDINGS IN ANIMAL EXPERIMENTS ON THE AETIOLOGY OF VIRAL HEPATITIS*

By

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(Received August 22, 1965)

Summary. A research team in the Institute of Hygiene, University Medical School, Budapest, has attempted to reproduce the results of BERTÓK *et al.* who described the experimental transmission of epidemic hepatitis virus into rats. In this paper histological findings in the liver of 34 rats used in the reproduction experiments are presented.

There was no pathological change in the liver of infected and control rats which received a normal synthetic diet. Identical changes were revealed in the liver of infected and non-infected animals fed on ethionine diet. A comparison of histological findings in livers obtained from human or canine infectious hepatitis with those in livers of rats treated with ethionine and subsequently infected with human hepatitis virus showed significant differences. It has been concluded that there are no such differences between ethionine-treated infected and only ethionine-treated rats' livers which would justify the assumption that the hepatitis virus is responsible for any specific change.

Human epidemic hepatitis has not yet been reproduced in animal experiments. In connection with this important problem BERTÓK [1], BERTÓK, FÁBER and PINTÉR [2] have described that, when pre-treated with ethionine, rats and guinea pigs become susceptible to infection with human hepatitis virus.

BERTÓK fed female rats and guinea pigs for 15 to 20 days on a diet containing 0.4 per cent ethionine, then infected the animals orally with the filtrate of the faeces of patients suffering from epidemic hepatitis. Animals that had not been given ethionine developed no symptoms. The majority of ethionine-treated guinea pigs and rats became ill, and 40 and 50 per cent, respectively, died. About 21 days after infection guinea pigs showed subfebrility lasting 2 to 3 days. Two-thirds of the affected animals developed jaundice. Control rats and guinea pigs that received ethionine but were not infected showed no signs of jaundice during life and after sacrifice.

The histological examinations of BERTÓK *et al.* revealed fatty vacuolar degeneration in the liver of ethionine-treated, not infected animals. The vacuoles compressed the nuclei of hepatic cells which became thus similar to fat cells. No inflammatory infiltration was observed. In the liver of animals treated with ethionine and then infected with faecal filtrate from patients with hepatitis, changes characteristic of acute human hepatitis were found. Further examina-

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tions excluded the possibility that ethionine *per se* would be capable of giving rise to the observed alterations.

In the Institute of Hygiene, University Medical School, Budapest, experiments have been performed to reproduce the findings of BERTÓK *et al.* Pathological examination of the liver of these animals was carried out in our institute.

Among 20 rats pre-treated with ethionine then infected with hepatitis virus 5 developed jaundice. Jaundice was observed also in 3 out of the 20 control (only ethionine-treated) animals.

We received livers of 34 animals out of which (1) 7 rats were fed on normal synthetic diet; (2) 10 rats were fed on normal diet then infected with human hepatitis virus; (3) 9 rats received ethionine treatment; (4) 8 rats were treated with ethionine then infected. The histological findings were as follows.

(1) and (2). No pathological changes were revealed in infected or non-infected rats fed on normal synthetic diet.

(3) Livers of rats fed on ethionine containing diet showed well-defined changes appearing mainly in the neighbourhood of branches of the portal vein. There was nuclear swelling in the epithelial cells of small bile ducts; in these cells chromatin was observed only along the nuclear membrane. In the neighbourhood of bile ducts a proliferation of cells was found, which, especially in the appearance of their nuclei, resembled epithelial cells lining the bile ducts. Such cells characterized by lightly stained nuclei were observed among hepatic cells situated at different distances from the bile ducts (Fig. 1). Deposit of fat globules was found mainly in periportal hepatic cells and also in phagocytes along the portal branches. The latter cells contained also PAS positive material and pigment giving positive Prussian blue reaction. In the liver of animals that developed jaundice, deposits of bile pigment were also noted.

Findings in ethionine-treated animals were characteristic. The hepatic cells, mainly their nuclei and nucleoli, were considerably enlarged. As shown by POPPER, DE LA HUERGA and YESINICK [7] and FARBER [3], the effect of ethionine on hepatic cells and especially on their nuclei is associated with hepatocarcinogenicity. For the development of a malignant tumour, of course, a much longer time is needed than the observation period in the present experiment. Necrosis of some hepatic cells could also be revealed. POPPER, DE LA HUERGA and KOCH-WESER [6] described centrolobular hepatic necrosis as a result of ethionine treatment. Under our experimental conditions such grave changes were encountered but occasionally.

(4) In rats pre-treated with ethionine then infected with hepatitis virus a proliferation of the epithelium of bile ducts was observed. The above mentioned cells characterized by lightly stained nuclei were also met with (Fig. 2). In this group, as well as in ethionine-treated not infected animals, the occurrence of fat in hepatic cells, PAS positive material and Prussian blue reaction along the portal branches were demonstrated. Enlargement of nuclei and nucleoli of

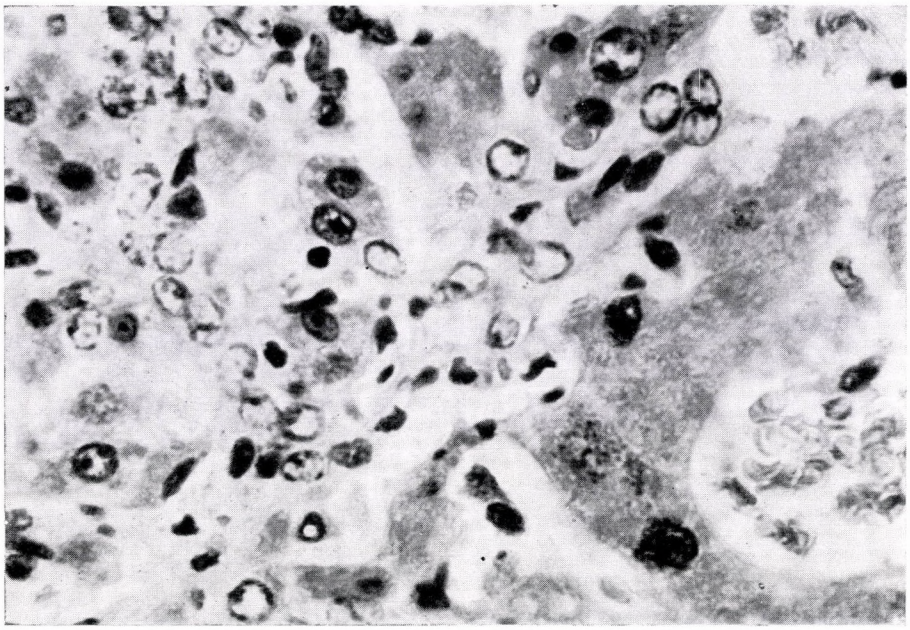

Fig. 1. Liver of ethionine-treated white rat. Among hepatic cells there is a proliferation of oval cells with lightly stained nuclei (bile duct epithelial cells). × 1120

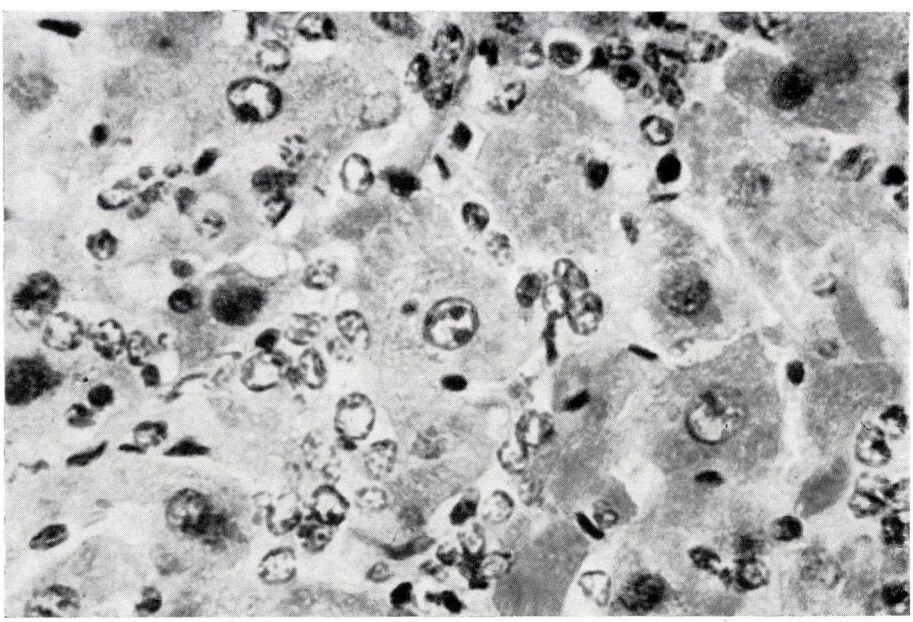

Fig. 2. Liver of rats treated with ethionine then infected with hepatitis virus is similar to those demonstrated in Fig. 1. Magnification, as in Fig. 1

hepatic cells was similar in both groups. Hepatocellular or centrolobular necrosis was not more definite in the infected than in the control group. Icteric changes, when present, were similar in the two groups.

Although an infectious disease may bring about different changes in man and animals, it is of interest to compare the above findings with those obtained

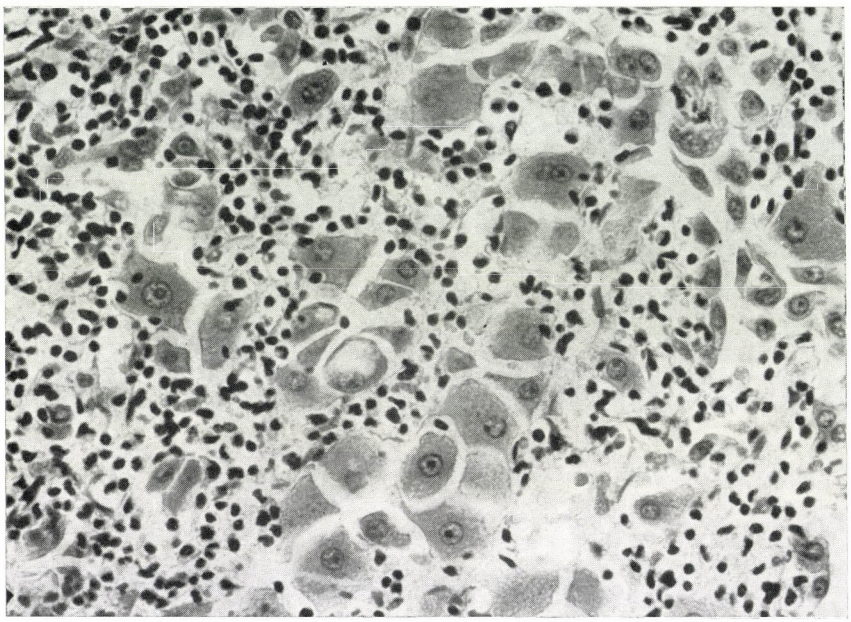

Fig. 3. Inflammatory hepatic changes in human hepatitis. × 400

in human and animal virus hepatitis. The fulminant form of human epidemic hepatitis may be fatal in 3 to 4 days. In the liver there is centrolobular or massive necrosis. In the interlobular connective tissue, infiltration with lymphocytes, plasma cells and histiocytes can be demonstrated. In subacute forms lasting 20 to 50 days the necroses are less extensive and beside inflammatory changes (Fig. 3), regeneration of hepatic cells and production of granulation tissue can be shown. In 1947, RUBARTH [8] described epidemic hepatitis in dogs. STÜNZI [9] found that changes in this disease were similar to those revealed in human hepatitis and yellow fever.

Comparison of changes observed in human and canine hepatitis with those revealed in the present experiments showed important differences. In the liver of ethionine-treated and infected rats necroses were shown only occasionally and there were signs of inflammation. BERTÓK *et al.* mentioned round cell infiltration in the liver of their animals treated with ethionine and subsequently

infected with hepatitis virus. In their microphotograph a proliferation of cells with lightly stained nuclei can be seen, which are similar to those revealed in the present studies in animals receiving only ethionine. In our opinion, in both ethionine-treated and ethionine-treated infected animals the most significant changes were a proliferation of bile capillaries and the enlargement of nuclei and nucleoli of hepatic cells.

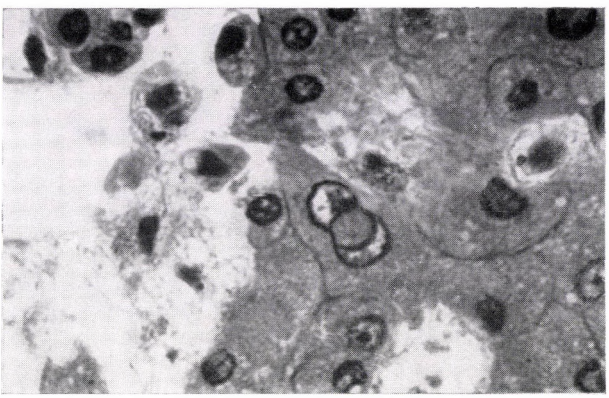

Fig. 4. Intranuclear inclusion bodies in human hepatitis. ×600

Nicolau [5] described intranuclear acidophilic inclusions in epidemic hepatitis. We have also demonstrated such inclusions in human hepatitis (Fig. 4). Stünzi [9] showed intranuclear inclusion bodies in hepatic cells of dogs with epidemic canine hepatitis. Kapp [4] also demonstrated such inclusions in dogs. In the liver of rats treated with ethionine and infected with hepatitis virus, no inclusion bodies could be found.

In conclusion, we have been unable to demonstrate differences between ethionine-treated and ethionine-treated infected rats that would be indicative of a specific effect exerted by hepatitis virus.

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AGAR-DIFFUSION METHOD FOR THE SCREENING OF ANTICANCER SUBSTANCES BY PHAGE INDUCTION

By

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Summary. A simple semi-quantitative agar-diffusion method suitable for mass examinations has been elaborated for the screening of anticancer substances by phage induction. The method is based on the following. A solid medium is infected with an appropriate mixture of the lysogenic and indicator strains; under the effect of active compounds, the number of plaques is significantly increased in the surroundings of the wells made in the agar plate.

LEIN, HEINEMANN and GOUREVITCH [1] have shown that certain antitumour substances induce phage production in lysogenic strains. HEINEMANN and HOWARD [2] and later SPECHT [3] presented data on the quantitative effects of various antitumour substances. MARJAI and IVÁNOVICS [4] obtained similar results with the phage- and megacin-induction of *B. megatherium*. The main point of both methods is that after induction in liquid medium the increase in phage production and megacin-titre is estimated on a solid medium with a suitable indicator strain. PRICE *et al.* [5] have modified HEINEMANN and HOWARD's method [2]; they adapted a streptomycin-dependent strain as lysogenic bacterium and separated the induction and phage-production process by streptomycin starvation. ENDO *et al.* [6] estimated the extent of lysis following induction by turbidimetry.

In our institute we searched for anticancer substances in Streptomyces fermentation broths first by the method of HEINEMANN and HOWARD [2]; later this laborious procedure has been developed into a rapid, semi-quantitative agar-diffusion method.

Materials and methods

Microorganisms. Escherichia coli K 12 λ-28 (lysogenic) and *Escherichia coli* C 600 (indicator).

Nutrient media. Broth containing 0.5 per cent peptone, with 2.0 per cent agar when used as base layer, or with 0.6 per cent agar when used for the infected layer.

Results and discussion

First, the culture of *E. coli* K 12 λ-28 was mixed in various proportions with that of the indicator strain, and with this mixed culture, added to a medium containing 0.6 per cent agar, was then infected the surface of Petri-dishes

filled with 20 ml of 2.0 per cent agar; 3 ml of the infected agar was used. It has been observed that when an appropriate dilution had been chosen sporadically located plaques appeared. If in these plates wells 10 mm in diameter were made and 0.1 ml of the solution of the anticancer substance was placed into them as for antibiotic determination, the number of plaques around the wells consider-

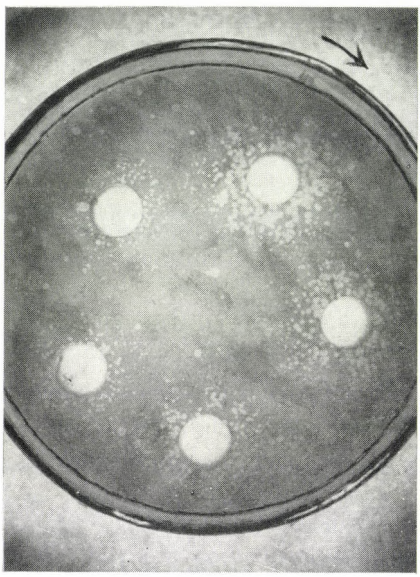

Fig. 1. Inductive effect of 0.37, 0.75, 1.5, 3.0 and 6.0 μg/ml of mitomycin C

ably increased. The clearest picture was obtained when the infected agar-layer contained $2-4 \times 10^3$ lysogenic and 2×10^7 indicator cells per ml. Fig. 1 shows such an experiment, where 0.1 ml of 0.37, 0.75, 1.5, 3.0 and 6.0 μg/ml mitomycin C solution was put into the wells, the plates were kept at room temperature for 2—3 hours, then incubated at 37°C for 16 hours. Along with the increase in mitomycin concentration extended the area where the number of plaques significantly increased as a result of induction. A precise reading was not feasible but, applying appropriate standard, the amount of the tested substance could be determined with 50 per cent accuracy.

The advantage of this diffusion screening method is that it is not laborious and thus well suitable for mass examinations. A further advantage is that a determination of the non-toxic concentration of the examined solution is not necessary, since in all cases where HEINEMANN and HOWARD's method yielded a positive result after dilution, the zone exhibiting diffuse plaques was outside the inhibition rings caused by the toxic substance. For the same reason, and this is an important point with fermentation broths where bactericidal

substances are also present, dilution of these antibiotics is unnecessary beside the inducing substance.

The use of a solid medium and mixed infection allowed the bioautography of paperchromatograms, which rendered possible the identification and separation of antitumour substances produced by various strains in the case of crude fermentation broths. In addition, the paper chromatographic results yielded information as to the chemical behaviour of the substance, which considerably facilitated the isolation procedure.

A disadvantage of the method is that the detection of non-diffusing substances, rarely encountered in fermentation broths produced by Streptomyces strains, is not feasible.

The sensitivity of HEINEMANN and HOWARD's method is 0.01 μg/ml of mitomycin C; the lower limit of our method is ten times higher, 0.1 μg/ml. However, as in these methods one can start only from 5—10-fold dilutions because of the interfering effect of crude fermentation broths, the sensitivity of the two method is the same. If the two methods are compared with regard to the disturbing effect of organic solvents mixing with water, our method proved 5—10 times less sensitive to the presence of substances, so that this disturbance roughly equalized the difference in sensitivity.

Our method gives only semi-quantitative results, since the diameter of the induction zone cannot be read precisely, but with induction in a liquid medium, exact quantitative results are achieved only with purified substances. Therefore, the method is especially suitable for following the purification procedure of the produced substances.

The turbidimetric method of ENDO *et al.* [6] is more cumbersome than the induction method on plates. Furthermore, substances causing lysis, which can be found in fermentation broths, may give erroneous results. Parallel examinations should therefore be made with not lysogenic strains.

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SHIGELLA FLEXNERI SEROVARIANTS CONTAINING COMBINATIONS OF TYPE ANTIGENS

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Summary. The serological, biochemical and pathogenic properties of nearly one hundred *Sh. flexneri* strains containing unusual combinations of type antigens are presented. The cultures contained also ε antigen and fell into two groups characterized by antigenic formulae S,I, (III), (IV): 6, (3, 4) and (S), (III) (IV) : 6, (3, 4). The majority of variants fermented mannitol and failed to produce indole. One strain fermented rhamnose. Rarely occurring sorbitol-splitting strains always produced indole. No association was found between serological and biochemical variations. In colonial morphology the cultures resembled *Sh. flexneri* 1b. They gave rise to keratoconjunctivitis shigellosa in guinea pigs.

In addition to sporadic dysentery, the new variants were responsible for smaller outbreaks. In geographical areas where these serovariants were encountered, the incidence of *Sh. flexneri* 1b infections was twice higher than in other areas. The new serovariants occurred at about the same frequency as rare *Sh. flexneri* types; a systematic survey in Szolnok county revealed them to comprise 2.7 per cent of all *Sh. flexneri* types.

Among *Sh. flexneri* strains originating from various parts of Hungary serovariants containing unusual combinations of type antigens were commonly met with. In the present paper serological and biochemical investigations into the new variants and an epidemiological survey carried out in Szolnok county, an area with approximately 500 000 inhabitants, are described.

Materials and methods

Serological examinations, in view of the large number of cultures, were performed with the slide agglutination technique. Absorbed sera were prepared as described by RAUSS [1] and by EWING [2]. One representative strain of each serological variant was examined by cross absorption tests. Preparation and absorption of sera were performed in our laboratory. Serum ε [3] was obtained together with the homologous strain W *6713* of MULCZYK and LACHOWITZ [4] from the Institute of Microbiology, University Medical School, Pécs. All sera were prepared and absorbed with antigens heated at 100° C for 2 hours.

Biochemical examinations were performed by routine methods described in references 1 and 2. Carbohydrate fermentation media were observed for 30 days. Beta-galactosidase activity was tested in addition to lactose fermentation in ONPG solution [5, 6]. Colonial morphology was examined on yeast extract agar at oblique light. Virulence of the cultures was tested by the keratoconjunctivitis reaction in guinea pigs [7].

Table I

Slide agglutination

Representative strain	No. of cases	Ewing's sera					
		I_{1a} (I)	I_{1b} (I + S)	III (H)	IV (K + IV_1 IV_2)	3, 4 (VIII)	6 (III)
97/65	2	++++	++++	++++	++++	++++	++++
498/64	73	—	++++	++++	++++	++++	++++
486/65	15	—	++++	++++	++++	++++	++++

Results

Results of slide agglutination are presented in Table I. Bracketed figures or letters under the symbols representing antigens of EWING's scheme stand for the corresponding antigen of RAUSS' scheme and, *vice versa*, those under the RAUSS factors mean the corresponding EWING antigens. As follows from differences in the methods of serum preparation and agglutination reactions, the corresponding factors in the two schemes are not necessarily identical. Absorbed sera I prepared from serum 1a and 1b were designated as I_{1a} and I_{1b}, respectively. Serum I_{1b} contained MADSEN's S factor.

Slide agglutination revealed several serovariants. Only two strains contained complete antigen I. These cultures were isolated from the same patient at a 1 month interval. Absorbed serum I prepared according to EWING from serum 1b agglutinated all strains because it contained agglutinins against the S factor. Sera prepared by EWING's and RAUSS' methods were different as to the reaction with antigen IV. EWING's serum IV agglutinated all strains. Neither of these strains contained the K factor of RAUSS' scheme which is characteristic of specific phase 4a strains. RAUSS' IV_1 IV_2 "group factor" serum agglutinated the majority of our strains. Different results were obtained with sera 3,4 (EWING) and the corresponding sera $VIII_2$, $VIII_{1, 2, 3}$ (RAUSS). On the basis of slide agglutination neither of our strains was identical with any known *Sh. flexneri* serotypes. The cultures were designated as type 1b—3b.

Absorption tests were carried out with strain 97/65 (positive in serum I) and with strain 498/64 (negative in serum I). The results are shown in Tables II and III. Of *Sh. flexneri* type strains, the highest titre agglutination in unabsorbed serum 97/65 was observed with type 1b, in serum 498/64 with type 3a. Aspecific agglutinations occurring with types 2b and 6 could be eliminated by absorption with *E. coli* 013.

The antigenic structure revealed by slide agglutination was confirmed by absorption tests. As expected from their different type antigen content, sera prepared with our variants were not completely absorbed by any type

of variants

Rauss's sera							
I	A(S)	H (III)	K	IV_1 IV_2	$VIII_2$	$VIII_{1',2',3}$	III (6)
(I)			(IV)		(3, 4)		
++++	++++	++++	−	−	−	−	++++
−	++++	++++	−	++++	−(+)	−(+)	++++
−	++++	++++	−	−	−(+)	−(+)	++++

strain *per se*. Details of these experiments were omitted from the Tables. All the agglutinins against type strains were removed only when absorption was carried out with two or more suitable serotypes. Sera absorbed in this manner still contained low titre agglutinins against strains 498/64 and 97/65 (1 : 40—1 : 80). Homologous agglutinins from serum 498/64 and homologous and some heterologous *Sh. flexneri* agglutinins from serum 97/65 were absorbed only by living cultures. When absorption was performed with suspensions heated at 100° C for 2 hours, the serum did not agglutinate heated suspensions but retained agglutinins against living homologous and some heterologous *Sh. flexneri* cultures. According to Table III, the latter strains shared antigens 3 and 4. The titre varied with types in the range of 1 : 80—1 : 320.

The presence of factors I and S in serum 97/65 was confirmed by the absorption test. The result of absorption of serum 498/64 did not, however, correspond to the result of slide agglutination. This serum was completely exhausted without type 1b, a mixture containing strains 3a, 4b and Y. Therefore S agglutinins could not be removed with strain 498/64 from serum 1b. Thus the antigenicity of the S factor present in strain 498/64 was lost, at least in the heated suspension. The two strains differed also in agglutinability: serum S agglutinated strain 97/65 at 1 : 160 and strain 498/64 at 1 : 40 dilution.

The presence of factors III and 6 was confirmed by various absorption tests. Type serum 3a was, however, not completely absorbed by living or heated strains even when the lacking antigens 7, 8, 9 were supplemented with type 2b or variant X. The remaining titre was not higher than 1 : 40.

According to their agglutinability in sera IV, 6 and 3, 4, our strains removed all agglutinins from sera 4b and Y. They were unable to absorb agglutinins 3, 4 from sera 2a and 4a and agglutinin IV (RAUSS's K) from serum specific phase 4a. Yet, strain 97/65 which was inagglutinable in serum IV_1, IV_2 (RAUSS) removed these agglutinins as well as strain 498/64, which gave a ++++ agglutination in this serum.

On the basis of absorption tests, the antigenic formulae of our strains can be expressed according to the international (EWING) designation as S, I, (III), (IV): 6, (3, 4) (strain 97/65) and (S), (III), (IV): 6, (3,4) (strain 498/64).

Table II

Antigenic analysis of

Serum	Absorbed by	Agglutination					
		498/64	97/65	1a	1b	2aA	2aB
498/64	—	12800	2560	640	320	160	160
	498/64	—	—	—	—	—	—
	97/65	++	(+)	—	—	—	—
	WZ 6713	++	(+)	—	—	—	—
	3a	++++	++++	(+)	++	—	++
	3a+1a	+++	+++	—	(+)	—	—
	3a+1a+4b	++	++	—	—	—	—
	3a+Y+4b	++	++	—	(+)	—	—
97/65	—	640	3200	640	1280	320	160
	498/64	—	+++	++	++	−××	−××
	97/65	—	−××	−××	−××	−××	−××
	WZ 6713	—	−××	−××	−××	−××	−××
	3a+1a+Y	+	++	−××	+++	—	−××
	3a+1b+Y	+	++	−××	−××	—	−××
	3a+1b+Y+4b	+	++	−××	−××	—	−××
	Living cultures						
	498/64	—	+++	+++	+++	—	—
	97/65	—	—	—	—	—	—
	WZ 6213	—	—	—	—	—	—
	3a+1b+Y+4b	—	—	—	—	—	—

−×× : agglutination with living bacteria but not with cultures heated for 2 hours.

In a serum prepared against thermolabile antigen ε, strains devoid of antigen I agglutinated more intensively than strains of the 97/65-type.

Biochemically our strains corresponded in character to the Shigella group. They failed to ferment sucrose, lactose, dulcitol, adonitol, inositol and salicin and did not split ONPG. All strains produced acid in arabinose, glucose and mannitol. Some sorbitol positive strains and one rhamnose positive culture were encountered. Sorbitol-splitting strains always produced indole. Neither strain produced urease and H_2S. All strains were methyl red positive and Voges-Proskauer, citrate, mucate and phenylalanine deaminase negative. MØLLER's decarboxylase reactions, with the exception of glutamic acid decarboxylase, were negative. There was no growth in KCN medium. The strains were non-motile in semisolid agar.

Our freshly isolated serovariants caused typical keratoconjunctivitis shigellosa in guinea pigs. Yeast extract agar plate cultures at transmitted light

strains 498/64 and 97/65g

2b	3a	4a sp	4a gr	4b	5	6 c	X	Y
1280	1280	80	160	640	80	1280	320	320
—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—
—	(+)	—	—	—	—	—	—	—
—	(+)	—	+++	++++	—	—	—	+++
—	—	—	—	+	—	—	—	—
—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—
320	320	80	640	640	160	640	160	320
—	—	—	—	—	—	—	—	—××
—	—	—	—	—	—	—	—	—××
—	—	—	—	—	—	—	—	—××
—	—	—	—	+	—	—	—	—××
—	—	—	—	+	—	—	—	—××
—	—	—	—	—	—	—	—	—××
—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—

often showed slightly refractive colonies with concentric rings [8]. Subcultures of these colonies were morphologically stable and avirulent with the keratoconjunctivitis test.

Investigation into the epidemiological characters of the new serovariants were carried out in Szolnok county. If regions where our variants occurred are grouped separately from those where it was absent (Table IV), it is seen that in the first group the frequency of *Sh. flexneri* 1b was twice higher than in the second group. As to incidence, our variants were among the more commonly occurring rare *Sh. flexneri* serotypes, even when the two groups are united. As to general morbidity, there was no difference between the two groups.

Table V shows the absolute number of our serovariants and serotype 1b encountered in various communities. A parallelism between the incidence of variants and type 1b was obvious, although not quite consistent. In several communities the variants occurred in larger numbers due to smaller outbreaks.

Table III

Antigenic analysis of strains 489/64 and 97/65

Serum	Absorbed by	Agglutination*	Result. Antigens in variants: + = present, − = absent, () = not complete or doubtful
1a	498/64	1b, 4a gr ++; 1a +++	I− 3, 4+
1b	498/64	97/5, 1a, 1b ++	I− 3, 4+ 6+
1b	498/64 + 1a	97/65, 1b ++	(S)
2a	498/64	97/65 +; 1a, 1b, 4a gr, Y ++; 2a, 2b +++	(3, 4)
3a	498/64	498/64, 2b, 5 +; 6, Y ++; 3a +++	6+ 7, 8, 9 −
3a	498/64 + X	3a +; 498/64 (+)	(III) 6+
4a sp	498/64	1a +; 5 ++; 4a sp, 4a gr, X, Y +++	(IV) (3, 4)
4a sp	498/64 + Y + X	4a sp ++++	(IV) K−
4a gr	498/64	1a, 4b, 5 +; X, Y ++; 4a gr +++	(IV) (3, 4)
4a gr	498/64 + Y + X	4b (+)	IV+ 6+
4b	498/64	4b (+)	IV+ 6+
Y	498/64	−	3, 4+
1a	97/65	1a (+)	I+ 3, 4+
1b	97/65	−	I+ S+ 3, 4+ 6+
2a	97/65	4a gr (+); Y ++; 2a, 2b +++	(3, 4)
3a	97/65	498/64, 2b, 5 +; 6, X ++; 3a +++	6+ 7, 8, 9 −
3a	97/65 + X	3a +	(III) 6+
4a sp	97/65	5 ++; 4a sp, 4a gr, X, Y+++	(IV) (3, 4)
4a sp	97/65 + Y + X	4a sp ++++	(IV) K−
4a gr	97/65	4b +; 5, X, Y ++; 4a gr+++	(IV) (3,4)
4a gr	97/65 + Y + X	4b (+)	IV+ 6+
4b	97/65	4b (+)	IV+ 6+
Y	97/65	−	3, 4+

* Agglutinations were performed in all sera with agar cultures of variants 97/65 and 498/64 and all *Sh. flexneri* types. In the Table only positive results have been included.

Table IV

Percentage distribution of serotypes in regions where variant 1b—3b was present (I) and absent (II)

Type	I	II	Total
1b—3b	6.67	—	2.67
1a	0.73	0.65	0.68
1b	25.90	12.96	18.17
2aA	31.20	50.12	42.56
2aB	0.14	0.73	0.48
2b	—	—	—
3a	26.30	22.44	24.00
4aA	2.16	5.18	3.93
4aB	0.61	0.48	0.53
4b	—	—	—
5	—	—	—
6	4.49	3.97	4.17
X	1.20	2.83	2.18
Y	0.60	0.64	0.63
Total	100.00	100.00	100.00

Table V

Number of cases associated with Sh. flexneri type 1b and variant 1b—3b

Community	Variant 1b—3b	Type 1b
A	$2_{(3)}$	6
B	$11^{c}_{(1, 2, 3)}$	2
C	$6^{c}_{(1, 2, 3)}$	57
D	$3_{(2, 3)}$	11
E	$9^{c}_{(1, 2, 3)}$	15
F	$2^{c}_{(3)}$	—
G	$2^{c}_{(3)}$	22
H	$9^{c}_{(2, 3)}$	72
I	$1_{(3)}$	—
J	$2^{c}_{(3)}$	1
K	$4^{c}_{(2, 3)}$	27
L	$1_{(2)}$	—
M	$1_{(2)}$	—
N	$2^{c}_{(1)}$	1

Abbreviations: Capital letters in column 1 represent various communities; ^{c} indicates smaller outbreaks (contact cases); indices $_{(1, 2, 3)}$ mean the occurrence of cases in the 1st, 2nd and 3rd year of observation.

Since the variants were met with throughout the three-year observation period, their occurrence was endemic. A spread of the variants to new areas was also noted.

Discussion

In the serological scheme of *Sh. flexneri*, constant combinations of group and type antigens determine the serotype of strains. The occurrence of unusual combinations is inconsistent with the principle of the system. Exceptions however, seem to exist.

McGUIRE and FLOYD [9] described that when fed to mice, *Sh. flexneri* 3a or Y strains disappeared from the intestine and gave way to *Sh. sonnei* II. OKADA, SASAKI and KARATO [10] transformed *Sh. flexneri* 1b into 3a by culturing the strain in the presence of serum I. GRZYBECK-HRYNCEVICZ [11] found that on intraperitoneal passages in mice *Sh. flexneri* lost its type antigen IV and gained antigen III instead. In this transformation group antigens were not affected. LACHOWITZ, MULCZYK *et al.* [12—14] transformed *Sh. flexneri* 1b by intraperitoneal passages in mice into a strain characterized by antigenic formula I, III : 3, 4, 6, then after further passages into a strain defined as III : 3, 4, 6. The latter variant contained MADSEN's antigen S and a thermolabile ε antigen. Similar mutants were isolated from 1b cultures after exposition to phage F_2 and from the faeces of patients. CARPENTER [15] noted similar strains as rarely occurring pathogens. In Hungary, in addition to our observations, some strains were isolated by VERTÉNYI [16].

Our strains 97/65 and 498/64 seem to be identical with LACHOWITZ and MULCZYK's first and second phases of artificially induced cultures. In antigenic structure strain 97/65 is obviously related to 1b; as to strain 498/64, the relationship is indicated only by the agglutinability in serum S. In colonial morphology [8] and sorbitol and indole negativity most strains also resembled serotype 1b. The conditions necessary for the appearance of factor IV in our variants are not known. The experience that in preparing absorbed sera according to EWING, side-agglutinins from serum I can be removed only with strain 4b, from serum IV only with strain 1b, indicates that types 1b and 4b are serologically related. The complete antigen IV was not present in our variants.

Usual antigens encountered in *Sh. flexneri* can be altered. This is indicated by the fact that some minor differences may occur in serologically regular strains. The thermolabile ε antigen can be regarded as such a serological variation. The unknown antigen, in the variants, the corresponding agglutinins of which cannot be removed with any *Sh. flexneri* type, may be a similar factor. The residual agglutinin in serum 97/65, which reacts with strains containing antigens 3, 4, may be due to a heat-stable antigen similar to antigen D shown by SLOPEK *et al.* [17] in *Sh. flexneri* 1b.

Although it seems probable, we have no proof that the appearance of variants in natural infection is a process similar to that observed under experimental conditions. Association of the variants with *Sh. flexneri* 1b cannot be confirmed statistically. In the faeces of a patient type 1b and variant 1b—3b occurred simultaneously. This finding was cautiously evaluated, as the faecal sample was examined in an other laboratory; moreover, simultaneous infections with type 1b could not be excluded. The epidemiological role of the variants is similar to that of less common *Sh. flexneri* serotypes.

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IMMUNOLOGICAL STUDIES ON SHIGELLOSIS BY THE MOUSE MODEL TECHNIQUE

I. ANTIINFECTIVE IMMUNITY OF ACTIVELY IMMUNIZED MICE

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Summary. In mice depleted of their coli flora by streptomycin treatment, oral administration of virulent *Sh. flexneri* 2a and 3 cells resulted in the symptomless excretion of these organisms. Excretion could be prevented by oral or parenteral active immunization. The immunity was type-specific and depended on the antigenic value of the vaccine and not on the virulence of organisms incorporated in the vaccine.

When administered orally, the immunizing effect of living avirulent cells was not superior to that of killed shigellae. The most effective oral vaccination was obtained with colloidal antigens.

Parenteral immunization was more effective than oral vaccination. Adsorbed antigens exerted the best immunizing effect. Immunity could be maintained with small oral doses of antigens.

Immunity manifesting itself with the prevention of shigella excretion by mice seems valuable for studying natural and artificial immunity to human dysentery and conforms to immunological principles revealed by other methods.

In previous studies [1] it has been shown that in mice depleted of their coli flora by streptomycin treatment, oral administration of shigellae results in a chronic symptomless excretion of the infective strain. Excretion can be induced with a small number of cells originating from Shigella colonies showing high refractivity under oblique light. According to COOPER [2] and KERE-KES [3], such colonies contain virulent organisms. Clones of non-refractive (avirulent [2, 3]) colonies did not give rise to chronic excretion even at doses containing several thousand millions of cells. Therefore, the capacity of inducing chronic excretion in mice as well as of giving rise to human dysentery is a property restricted to virulent strains. It has also been shown that successful mouse infection is associated with the penetrating power of the organism through the intestinal epithelium. The invasion by shigellae is limited to the lymphatic system of the intestinal wall and the organisms reach the mesenterial lymph glands only exceptionally.

These observations indicated that human and mouse shigelloses have many common characters. It has therefore been studied whether symptomless chronic Shigella carrier mice are suitable for immunological experiments in which the immune reaction is expressed by the prevention of Shigella excretion. The present paper discusses the results of active immunization.

Material and methods

Antigens and vaccines. For active immunization living cells or suspensions killed with 0.3 per cent formalin were used. The suspensions were adjusted to the density of the Human substandard of the N. I. H. 10T standard. Boivin extracts used as colloidal antigens were standardized by the haemagglutination inhibition technique [4]. Adsorbed vaccines were prepared by mixing Boivin antigens with preformed Willstätter C-γ-type $Al(OH)_3$ gel as modified by RÉTHY [5]. The adsorbent-antigen ratio was 2.5 mg/ml.

Experimental animals. Albino mice (strain Human) weighing 20 to 25 g were used. Two days before infection the animals were placed singly in sterile glass jars or in groups in well-closing sterile boxes containing sterile litter. The animals received autoclaved food. Two days before infection every mouse was given a daily dose of 50 mg streptomycin by the oral route. Elimination of the coli flora was checked by culturing rectal scrapings on Endo medium. During the experiments a daily dose of approximately 1 mg streptomycin was mixed into the food. Aseptic conditions were rigorously maintained.

Active immunization was carried out with subcutaneous injections of single or graded doses of the antigen. The latter were given at one week intervals. The animals were infected 10 days after the last injection. Oral immunization was performed generally with 5 graded doses administered at 3 day intervals. Oral doses were 10 to 20 times larger than parenteral doses.

Infection of mice. Virulent mutants selected on the basis of refractivity were used. Most experiments were performed with virulent *Sh. flexneri* strains "V" (type 2a) and RV_I (Type 3). Both strains were resistant to 1000 μg/ml streptomycin. After checking the refractivity, 18 hour agar plate colonies were suspended and diluted serially. Infection of the animals was carried out orally with 0.5 ml dilution containing approximately 5 cells. This dose corresponded to the 50 per cent infective dose (ID_{50}). The actual bacterial count was simultaneously determined by the pour plate technique.

Detection of Shigella excretion. Five days after infection rectal scrapings were streaked onto eosin-methylene blue agar. Cultural examination was occasionally repeated. Shigellaform colonies were examined by slide agglutination with absorbed sera.

Statistical analysis. Prevention of Shigella excretion in immunized mice was always compared with results obtained in a non-vaccinated control group of animals. The ratio of excreters in the immunized and control groups was expressed in per cents (ID_n). When graded doses were used for immunization, the 50 per cent effective dose (ED_{50}) was calculated as described by KÄRBER [6]. Significance at 5 per cent limits was estimated by the χ^2 test.

Results

Virulence of strains. In selecting the test organisms, determination of their virulence was essential for obtaining congruent and reproducible results. In some experiments we succeeded in demonstrating immunity by use of a heterogeneous population; from the variable results, however, no definite conclusions could be drawn. These preliminary experiments indicated that inhibition of persistent shigella excretion in mice is adequate for immunity studies and, on the other hand, that superinfection should be performed with the minimal infecting dose.

Results obtained by selecting strains on the basis of virulence revealed that the incongruency of previous findings was due to the variable ratio of virulent and avirulent cells in unselected cultures. Thus, successful infection depended on the number of virulent cells in the heterologous population.

Preliminary experiment. The result of one of our basic experiments is presented in Table I. Groups of mice containing 9—10 animals each were immunized with graded doses of adsorbed vaccine and with Boivin extract. The

Table I

Immunity of vaccinated mice
(Challenging dose: *Sh. flexneri* 3, "RV_I" 10 cells)

Vaccine and mode of administration	Dose (ml)	Relative number of excreters				χ^2
		5	11	18	Average	
		days after infection				
Adsorbed Boivin, 2 subcutaneous doses	0.5	ID_{27}	ID_{18}	ID_{18}	ID_{21}	8.953
	0.1	ID_{40}	ID_{40}	ID_{30}	ID_{37}	4.748
	0.02	ID_{37}	ID_{50}	ID_{37}	ID_{41}	3.517
Colloidal Boivin, 5 oral doses	5.0	ID_{14}	ID_{14}	ID_{14}	ID_{14}	8.526
	1.0	ID_{63}	ID_{54}	ID_{45}	ID_{57}	1.190
	0.2	ID_{81}	ID_{72}	ID_{63}	ID_{73}	0.189
Control (non-immunized)		ID_{86}	ID_{80}	ID_{93}	ID_{87}	

former was given in 2 subcutaneous injections, the latter in 5 oral doses administered at 3 day intervals. Challenge was performed orally with 10 cells of virulent *Sh. flexneri* 3 strain RV_I. As shown by repeated cultural examinations, animals in the control (non-immunized) group became excreters in 80 to 93 (average 87) per cent. A definite and statistically significant immunity was observed in mice receiving both parenteral and oral vaccination. It is seen that there was an association between antigen dose and immune response. Although the effectiveness of the two vaccination methods could not be compared exactly, it was evident that the adsorbed vaccine was more effective than oral immunization with colloidal antigen.

Specificity of immunity. Our preliminary experiments have shown that the mouse model is suitable for studying immunological problems. Before continuing these experiments it seemed necessary to confirm the specificity of the immune response manifesting itself with the inhibition of Shigella excretion. Therefore groups of mice were immunized parenterally with 0.1 ml adsorbed and orally with 1.0 ml colloidal vaccines prepared from *Sh. flexneri* types 2a and 3 and from *Sh. sonnei.* The animals were then infected with virulent cells of *Sh. flexneri* 2a strain "V". The results are presented in Table II.

It can be seen that there was a significant difference between animals immunized with the homologous strain and animals of the control groups. As only the homologous vaccine gave rise to a significant immune response, immunity obtained in the mouse model was specific, or more exactly, type-specific.

Protective value of different types of vaccines. In further experiments the immunogenic capacity of various antigens was compared. The experiments were carried out in two parts; in the first part (Table III) colloidal Boivin

Table II

Specificity of immune response
(Challenging dose: *Sh. flexneri* 2a "V", 7 cells)

Vaccine and mode of administration	Dose (ml)	Relative number of excreters immunized with			Control
		Sh. flexneri 2a (homologous)	*Sh. flexneri* 3 (heterologous)	*Sh. sonnei* (heterologous)	
Adsorbed Boivin, 2 subcutaneous doses	0.1	ID_{20} (3.333)*	ID_{40} (0.800)	ID_{60} (0.000)	ID_{60}
Colloidal Boivin, 5 oral doses	1.0	ID_{10} (5.494)	ID_{50} (0.202)	ID_{60} (0.000)	ID_{60}

The framed area indicates significant immunity against the homologous strain
* χ^2 values

Table III

Protective effect of various vaccines
(Challenging dose: *Sh. flexneri* 2a "V", 6 cells)

Vaccine and mode of administration	Dose (ml or No. of cells)	Relative number of excreters 6 days after infection	ED_{50}
Colloidal Boivin, 5 oral doses	0.04	ID_{70} (0.266)*	
	0.2	ID_{50} (1.700)	
	1.0	ID_{20} (7.200)	0.2 ml (2.5×10^8 cells)
	5.0	ID_{10} (9.898)	
Formalinized cells, 5 oral doses	4×10^8	ID_{55} (1.309)	
	2×10^9	ID_{44} (2.572)	
	10^{10}	ID_{33} (4.231)	2×10^9 cells
	5×10^{10}	ID_{20} (7.200)	
Living avirulent cells, 5 oral doses	4×10^8	ID_{60} (0.952)	
	2×10^9	ID_{40} (3.333)	
	10^{10}	ID_{30} (5.050)	1.9×10^9 cells
	5×10^{10}	ID_{20} (7.200)	
Control (non-immunized)		ID_{80}	

* χ^2 values

extract, living bacteria and formalinized bacteria were given by the oral route.

From Table III it is obvious that suitable antigen doses produced a significant immunity. The protective effect of the vaccine increased parallel with the dose of antigen. When the immunogenicity of various vaccines is compared, it is seen that the immunizing effect of living and formalinized cells was similar ($ED_{50} = 2 \times 10^9$ cells). The corresponding value for the Boivin antigen was 0.2 ml. As demonstrated in a preliminary haemagglutination inhibition test, this amount corresponded to 2.5×10^8 cells. Therefore, the protective effect of the colloidal antigen was approximately 10 times higher than that of the two other vaccines.

In the second part of experiments the effect of oral Boivin antigen was compared with subcutaneously given adsorbed Boivin antigen and formalinized vaccine (Table IV).

Due to the low infective dose (4 cells), the inhibition of excretion was very definite and the protective effect of even the smallest antigen doses was weaker than the ED_{50}. The superiority of parenteral adsorbed vaccine is ob-

Table IV

Protective effect of various vaccines
(Challenging dose: *Sh. flexneri* 2a "V", 4 cells)

Vaccine and mode of administration	Dose (ml and cell equivalent or No. of cells)	Relative number of excreters, average of 6 and 12 day results after infection
Colloidal Boivin, 5 oral doses	0.2 (2.5×10^8)	ID_{28} (5.783)*
	1.0 (1.25×10^9)	ID_{11} (10.243)
	5.0 (6.25×10^9)	ID_{05} (13.081)
Adsorbed Boivin, 2 subcutaneous doses	0.01 (1.25×10^7)	ID_{25} (6.857)
	0.05 (6.25×10^7)	ID_{10} (11. 316)
	0.25 (3.12×10^8)	ID_{06} (12.030)
Formalinized cells, 2 subcutaneous doses	2×10^9	ID_{15} (9.696)
	10^{10}	ID_{10} (11.316)
	5×10^{10}	ID_0 (15.000)
Control (non-immunized)		ID_{75}

* χ^2 values

vious. As this antigen yielded about the same effect as 20-fold amounts of oral Boivin extract, it is justified to state that its protective effect was 20 times higher. According to cell count equivalence, the weakest effect was exerted by parenteral formalinized vaccine.

Immunogenicity of virulent and avirulent mutants. It was interesting to compare the immunogenicity of vaccines prepared from selected virulent and avirulent mutants. Virulent ("V") and avirulent ("AV") mutants of *Sh. flexneri* 2a were used for the preparation of formalinized vaccine and Boivin extract. The antigens were standardized by the haemagglutination inhibition test. One ml of Boivin extract prepared from the avirulent culture was equivalent to 0.75 ml Boivin extract of virulent bacteria. The equivalent inhibiting titre of the corpuscular vaccines was 10^9 cells. Five doses of the above amounts of extracts were given orally. The corpuscular vaccines were administered in 2 subcutaneous doses. The results are shown in Table V.

Table V

Protective effect of corpuscular vaccines and antigen extracts prepared from virulent and avirulent strains
(Challenging dose: *Sh. flexneri* 2a "V", 5 cells)

Vaccine and mode of administration	Average relative number of excreters	χ^2 values between virulent and avirulent vaccines
Sh. flexneri 2a "V"* Boivin, 5 oral doses of 0.75 ml each	ID_{33}	0.977 ($0.5>P>0.3$)
Sh. flexneri 2a "AV"* Boivin, 5 oral doses of 1.0 ml each	ID_{25}	
Sh. flexneri 2a "V" formalinized cells, 2 subcutaneous doses	ID_{20}	0.143 ($P\sim0.7$)
Sh. flexneri 2a "AV" formalinized cells, 2 subcutaneous doses	ID_{25}	
Control (non-immunized)	ID_{85}	

Vaccines prepared from avirulent and virulent strains were standardized by the haemagglutination inhibition test. Immunization was performed with equivalent amounts.
* V = virulent, AV = avirulent

All types of vaccines produced significant immunity as compared to the control. There was no difference in the protective effect of suspensions or extracts prepared from virulent and avirulent mutants.

Duration of immunity. Results are summarized in Table VI. Two groups of mice, each consisting of 80 animals were immunized subcutaneously with adsorbed Boivin antigen. Animals in one of these groups received additional

weekly doses of 0.05 ml Boivin antigen orally. After streptomycin treatment at weekly intervals 10 mice from each immunized group and from the control (streptomycin-treated, not immunized) group were infected orally with 4 to 9 virulent cells. As shown in Table VI, "basal immunity" developing after immu-

Table VI

Duration of immunity in mice immunized subcutaneously with adsorbed vaccine then revaccinated orally
(Challenging dose: *Sh. flexneri 2a* "V", 4 to 9 cells)

Days after the second dose of precipitated vaccine	Average relative number of excreters		
	Immunized subcutaneously then revaccinated orally	Immunized only subcutaneously (not revaccinated)	Not immunized
7	.	ID_{20} (7.200)*	ID_{80}
14	ID_{10} (5.494)	ID_{10} (5.494)	ID_{60}
21	ID_{20} (7.200)	ID_{30} (5.050)	ID_{80}
28	ID_{20} (5.050)	ID_{20} (5.050)	ID_{70}
35	ID_{20} (7.200)	ID_{40} (3.333)	ID_{80}
42	ID_{10} (9.898)	ID_{50} (1.978)	ID_{80}
49	ID_{20} (5.050)	ID_{60} (0.219)	ID_{70}

First immunization was carried out with two doses of 0.1 ml adsorbed Boivin extract at an interval of one week. Revaccination was performed orally with weekly doses of 0.05 ml Boivin extract.

* χ^2 Values; □ = significant, ⬚ = nearly significant results.

nization with the adsorbed vaccine lasted approximately for 4 weeks. After 5 weeks the difference from the control was still close to the level of significance. Examinations carried out on the 42nd and 49th days revealed that immunity had been lost. On the other hand, by weekly oral vaccination with small amounts of antigen (0.25 ED_{50}), immunity was maintained at a constant level throughout the observation period.

Discussion

Active immunization experiments have shown that our mouse model based on the symptomless excretion of shigellae is suitable for studying artificial immunity against dysentery. A criterium of successful experiments is the selection of virulent mutants, as congruency and reproducibility of the results can be secured only if infecting doses consisting of uniformly virulent cells are used. Active immunization prevented the development of Shigella excretion, in other words, it brought about an antiinfective immunity. As Shigella excretion in mice is symptomless, the model experiment does not allow the observation of "clinical immunity". According to data in the literature, immunity against bacillary dysentery is characterized by a "clinical immunity" which inhibits the clinical manifestation of the disease. A classical example of this is TROICKIJ's [7] observation on monkeys showing clinical immunity, but becoming Shigella excreters after reinfection. In our opinion this observation explains why the mouse, even when it has been given large amounts of antigen, is immune against small challenging doses only. A higher degree of immunity is probable for preventing excretion than for protecting against the development of clinical symptoms. There is, however, no reason to assume that a difference other than quantitative exists between the two types of immunity. Consequently, it is justified to regard our model as a very rigorous test of immunity. TROICKIJ [7] has characterized protection against dysentery as a fairly insubstantial immunity.

Our investigations have shown that immunity can be produced by both parenteral and oral vaccination. The degree of immunity corresponds, although not according to a linear relationship, to the dosage of the vaccine. The immunity is definitely type-specific.

In comparing the immunogenic effect of various vaccine types, the results were similar to those of previous experiments [9—12]. An important observation is that oral immunization by living avirulent bacteria confers no higher degree of immunity than that produced by formalin-killed microorganisms. The finding is due to the fact that avirulent cells (*Sh. flexneri* 2a "AV") were incapable of penetrating into the intestinal mucosa. Thus it follows that this finding cannot be valid for avirulent microorganisms which maintained their penetrating (invasive) capacity.

After completion of the present study, FORMAL *et al.* [15] have published a paper confirming experimentally the above consideration. In monkeys immunized with an avirulent mutant incapable of penetration, these authors demonstrated an immunity equivalent to that obtained with killed bacteria. In contrast, an *E. coli-Sh. flexneri*-hybrid that had retained its epithelial penetrating capacity and was avirulent only because it failed to multiply in tissues, gave rise to an effective clinical immunity.

As compared with corpuscular vaccines, orally administered colloidal Boivin antigen was approximately 10 times more effective (Table III). This result corresponds in order of magnitude to the 3 to 4-fold difference obtained with the mucin technique [9]. As shown in Table IV, the effectiveness of parenterally given adsorbed vaccine was about 20 times higher than that of oral, unadsorbed antigenic extract. In our previous experiments [9] performed with the mucin technique, the difference in the effectiveness of these vaccines was similar in order (30-fold). It should be noted that our adsorbed vaccine, which had proved most effective in laboratory experiments [10, 11] and human trials [12], was also the most effective among all vaccines when examined in the model test.

Data included in Table V reveal that there is no association between virulence factor(s) and protective LPS antigens.

As regards the duration of immunity, there are two practical questions: is an effective immunity obtained with adsorbed vaccine persistent, and, can the immunity be maintained with small oral antigen doses? As shown in Table VI, adsorbed vaccine maintains an effective immunity for a period of approximately 1 month; oral revaccination with 0.05 ml (0.25 ED_{50}) amounts of Boivin extract prolongs the immunity for the whole observation period (50 days).

From our results it may be concluded that the phenomenon of immunity is well demonstrated by our method imitating natural infection. Principles of immunity observed with other methods, such as the association of the effectiveness of vaccination with type-specificity, amount and nature of the antigen, route of dosage, antigenic value but not virulence, can also be shown with the model experiment.

It remains to be discussed how our results could be used for elucidating problems of human dysentery. As mentioned in the introduction, the pathomechanism of mouse shigellosis resembles that of the human disease [1].

FORMAL *et al.* who worked with starved guinea pigs [8, 15], concluded as to the pathomechanism of dysentery that the causative agent penetrated through the intestinal epithelium and multiplied in the lamina propria. In our opinion the same occurs in the mouse model. Penetration through the epithelium into the lamina propria is confirmed by the presence of shigellae in the intestinal lymphatic system; the ability of the organism to multiply in tissues is indicated by the persistence of excretion.

Thus the ability to local invasion and multiplication is a predominating factor in model shigellosis including keratoconjunctivitis shigellosa [13] and intracellular multiplication in tissue cultures [14]. Thus, the analogy with human dysentery is obvious and therefore both in humans and animals an "anti-invasive" immunity is probably involved. It may be assumed that the mechanism of immunity is identical in natural and in model infection and thus our observations can be applied cautiously to artificial immunity of humans.

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IMMUNOLOGICAL STUDIES ON SHIGELLOSIS BY THE MOUSE MODEL TECHNIQUE

II. ANTIINFECTIVE IMMUNITY OF PASSIVELY IMMUNIZED MICE

By

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Summary. Symptomless shigella excretion can be prevented by subcutaneous or oral passive immunization of mice with serum or copro-antibodies. The humoral factor of immunity is decisive in the immune mechanism of mouse shigellosis.

Serum and copro-antibodies are equivalent in protective effect. Apart from their localization, there is no qualitative difference between the two kinds of antibody.

The best effect is yielded by oral passive immunization; the importance in immunity of high antibody concentrations in the intestine is obvious from the pathomechanism of shigellosis.

In previous papers [1, 2] we have shown that oral infection with virulent mutants of shigella strains of mice depleted of their coli flora by streptomycin treatment results in a chronic symptomless excretion of the infective agent. Except for pathological changes, shigella infection of mice shows many analogous features with human dysentery as regards epithelial penetrating ability, limited lymphogenic invasiveness and excretion of shigellae, production of copro-antibodies, etc. Thus our model has been termed "mouse shigellosis".

We have concluded that the mouse model is suitable for studying active immunity [2]. It has been shown that the immune response characterized by the prevention of shigella excretion is well defined and conforms to known principles of dysentery immunity. The present paper gives an account of passive immunization studies on humoral immunity.

Materials and methods

Care, streptomycin treatment, infection, detection of shigella excretion of the animals. and statistical analyses were carried out as described in reference 2.

Immune sera and intestinal extracts. Passive immunization experiments were performed partly with hyperimmune rabbit sera, partly with sera of chronic carrier mice which had been bled through the retrobulbar venous plexus [3]. Intestinal extracts containing copro-antibodies were prepared from the intestinal tract of chronic carrier mice sacrificed by bleeding. The intestine was ground with quartz sand then suspended in saline at g/ml amounts and filtered through glass filter after storage at 4° C overnight.

Hyperimmune rabbit sera were preserved with 0.5 per cent phenol and stored at 5° C. Mouse sera and intestinal extracts were kept at —20° C.

Passive immunization was performed with immune sera and intestinal extracts diluted 1:5 or 1:10. At different intervals, 2 to 7 subcutaneous injections were given. Oral immunization was carried out similarly; one oral dose was always given simultaneously with the infecting bacteria.

Results

The importance of humoral immunity in mouse shigellosis was studied in passive immunization experiments. As those of active immunization, the results of passive immunization were reproducible only when the animals had been infected with selected virulent mutants. As shown in preliminary experiments, an appreciable immunity manifesting itself in the inhibition of shigella excretion developed only when the immune serum was given in large doses and the number of infecting bacteria was less than the 100 per cent infecting dose. The association of immune effect and challenging dose is presented in Table I.

Table I

Influence of different serum and infective doses on immunity
(Homologous hyperimmune rabbit serum orally; challenging organism: *Sh. flexneri 2a* "V")

Serum doses (ml)*	Relative number of excreters among animals infected with		
	6 cells	9 cells	24 cells
0.1	ID_{10} (7.500)**	ID_{30} (5.050)	ID_{50} (3.809)**
0.02	ID_{20} (5.050)	ID_{40} (3.333)	ID_{60} (2.400)
0.004	ID_{10} (1.818)	ID_{60} (0.952)	ID_{80} (0.392)
Control	ID_{70}	ID_{80}	ID_{90}

*The serum was administered in 5 doses, one dose on each of 2 consecutive days before and after infection and one dose on the day of infection

** χ^2 values.

☐ significant, ⬚ nearly significant, difference as related to the control.

In three experiments, groups of mice containing 10 animals each were protected with hyperimmune rabbit serum. The doses for the three groups were so chosen as to form a 5-fold geometrical progression: 0.1, 0.02 and 0.004 ml. The first and second doses of serum were given on each of two consecutive days before infection, the third simultaneously with the infecting bacteria, the fourth and fifth on each of two consecutive days after infection. The challenging doses as estimated by plate counting contained 6, 9 and 24 cells. These infecting doses caused excretion in 7, 8, and 9, out of 10 mice included in the respective control groups (ID_{70}, ID_{80} and ID_{90}). Table I shows that 0.1 and 0.02 ml serum doses significantly protected the animals against 6 cells. A significant protective effect against 9 cells was produced only by 0.1 ml serum. The immune response yielded by 0.02 ml serum only approached the 5 per cent

limit. Serum in 0.1 ml doses produced less significant immunity against an infecting dose of 24 cells. Thus, valuable immunity can be obtained only when large immunizing doses and small challenging doses are used. The data also show the parallel decrease of the intensity of immune response with the graded decrease of serum doses.

It has been shown previously [4] that serum and intestinal extract of chronic excreter mice contain protective antibodies against shigellae injected intraperitoneally in mucin. In the present study the protective effect of serum and intestinal extract of mice excreting shigellae for 3 weeks has therefore been examined. The antiinfective effect of subcutaneously and orally given sera is presented in Table II.

It is seen that the serum of chronic carrier mice displayed immunity after both oral and subcutaneous administration.

In Table III the passive immunizing effect of intestinal extract is demonstrated. Groups of mice containing 10 animals each were immunized by the subcutaneous, oral and combined subcutaneous-oral routes with 7 large doses of intestinal extract. The data indicate that copro-antibodies exert a good protective effect. The degree of immunity was significant with any of the used immunizing methods.

Tables II and III reveal that oral immunization tends to be more effective. In subsequent experiments a comparison was made between oral and subcutaneous immunization. In addition, the relative protective effect of serum and copro-antibodies and the result of immunization with 2 and 5 doses of serum and intestinal extract was compared. Results are summarized in Table IV.

Copro-antibodies were seemingly more effective than serum antibodies, Subcutaneous immunization was somewhat less effective than oral immuniza-

Table II

Protective effect of serum of chronic carrier mice
(Challenging dose, *Sh. flexneri* 2a "V", 4 cells)

Mode of administration	Relative number of excreters			χ^2
	4 days after infection	9 days after infection	Average	
3 subcutaneous doses of 0.1 ml each*	ID_{10}	ID_{10}	ID_{10}	10.332 $P<0.01$
3 oral doses of 0.1 ml each	ID_{10}	ID_{0}	ID_{5}	11.025 $P< 0.001$
Control	ID_{77}	ID_{88}	ID_{83}	.

* Serum of mice which had been excreting shigellae for 3 weeks was given in 3 doses: one dose on each day before and after infection and one on the day of infection.

Table III

Protective effect of intestinal extract (copro-antibodies) of chronic carrier mice
(Challenging dose, *Sh. flexneri* 2a "V", 5 cells)

Mode of administration	Relative number of excreters			χ^2
	5 days after infection	12 days after infection	Average	
7 subcutaneous doses of 0.1 ml each*	ID_{20}	ID_{20}	ID_{20}	5.050
7 oral doses of 0.1 ml each*	ID_{10}	ID_{10}	ID_{10}	7.500
7 subcutaneous and oral doses of 0.1 ml each**	ID_{10}	ID_{20}	ID_{15}	5.625
Control	ID_{70}	ID_{70}	ID_{70}	·

* Intestinal extract was given in 7 subcutaneous or oral doses: one dose on each of 2 consecutive days before and on each of 3 consecutive days after infection, and two doses on the day of infection.

** Intestinal extract was given in 7 subcutaneous doses (one dose on each of 2 consecutive days before and on each of 3 consecutive days after infection) and in 2 oral doses (one on the day of infection and one on the next day).

Table IV

Origin and mode of administration of antibody, and the effectiveness of immunization
(Challenging dose, *Sh. flexneri* 2a "V", 6 cells)

Origin of antibody	Mode of administration	Number of doses	Average relative number of excreters	χ^2
Serum	Subcutaneous	5	ID_{30}	8.120
		2	ID_{50}	2.666
	Oral	5	ID_{25}	10.000
	Subcutaneous + oral	5	ID_{20}	12.130
Intestine	Subcutaneous	5	ID_{25}	10.000
		5	ID_{15}	14.482
	Oral	2	ID_{50}	2.666
	Subcutaneous + oral	5	ID_{25}	10.000
Control			ID_{75}	·

When given in 5 doses, serum or intestinal extract was administered on two consecutive days before and on two consecutive days after infection and on the day of infection. When given in 2 doses, the antibody was administered on the day before infection and on the day of infection. When the same animals received both parenteral and oral immunization, one subcutaneous injection was given on each of two consecutive days before infection and on the second day after infection; oral doses were given on the day of infection and on the next day.

tion. It is also evident that 2 antibody doses were inferior to 5 doses. Under the given experimental conditions 2 doses yielded no significant immune response on either subcutaneous or oral administration.

In view of these results it was desirable to compare the relative protective effect of copro and serum antibodies under quantitative conditions. Accordingly, the following experiment was performed. Graded (0.05, 0.005, 0.0005 ml) amounts of serum and intestinal extract were injected subcutaneously to groups of mice. The animals were infected intraperitoneally with a 5 per cent mucin suspension of the homologous organism representing 500 LD_{50}. The serum and intestinal extract exerted a protective effect of $ED_{50} = 0.05$ ml for serum and $ED_{50} = 0.016$ ml for intestinal extract.

The two materials were brought to equivalence by diluting the intestinal extract then groups of mice containing 10 animals each were immunized with 6 doses administered by the subcutaneous, oral and combined subcutaneous-oral routes. Results are summarized in Table V.

Table V

Protective effect of standardized hyperimmune rabbit serum and intestinal extract of chronic carrier mice

(Challenging dose, *Sh. flexneri* 2a "V", 7 cells)

Origin of antibody	Mode of administration	Number of doses	Average relative number of excreters	χ^2
Serum	Subcutaneous	6	ID_{30}	3.333
	Oral	6	ID_{10}	9.898
	Subcutaneous + oral	3 + 3	ID_{20}	7.200
Intestine	Subcutaneous	6	ID_{30}	5.050
	Oral	6	ID_{10}	9.898
	Subcutaneous + oral	3 + 3	ID_{20}	7.200
Serum + Intestine	Subcutaneous + Oral	3 + 3	ID_{20}	7.200
Control			ID_{80}	.

Rabbit serum and intestinal extract were previously brought to equivalence by passive mouse protection test performed with the mucin technique. Serum or copro-antibodies were administered in 6 subcutaneous or oral doses: one dose on each of 2 consecutive days before and after infection and two doses on the day of infection. When the same animals received both parenteral and oral immunization, one oral dose was given 2 days before infection, one subcutaneous dose on the day before infection, one subcutaneous and one oral dose on the day of infection, one subcutaneous dose on the day after infection, and one oral dose 2 days after infection.

It is evident that, when brought to equivalence, copro and serum antibodies exerted the same degree of protective effect. Data shown in Table V definitely confirmed the previous observation that oral administration of both serum and copro-antibodies was superior to subcutaneous immunization. As it might be expected from this finding, combined subcutaneous-oral administration yielded an immune response closer in degree to that of oral immunization.

Discussion

By means of the "mouse-shigellosis" model it has been shown that shigella excretion can be inhibited by passive immunization. The main purpose of the present experiments was to reveal the character of immunity against shigellosis. The findings were clearly indicative of the considerable part played by humoral factors in the immune mechanism of "mouse-shigellosis". It should be considered that active immunization produces no higher protective effect than passive immunization; in other words, similarly to passive immunization, vaccination with large doses produces antiinfective immunity only against a challenging dose not exceeding the minimal infective dose. From this finding it follows that the humoral factor plays the decisive part in both active and passive immunity. The present experiments have confirmed our previous finding in that mice excreting shigellae produce high titre protective serum and copro-antibodies.

Comparison of the protective effect of serum and copro-antibodies under standardized conditions has shown that there is no qualitative difference between serum and copro-antibodies. This result is obvious and may be expected in view of the conception of the unity of body functions distinguishing serum and copro-antibodies according to their localization only. It is probable that competent cells of the intestinal tract are primarily responsible for the production of antibodies, a larger amount of which acts as copro-antibodies, while the rest gains entrance into the circulation and appears as serum antibodies. In shigella infection, a mobilization of the total immune system cannot, of course, be excluded. That this happens, is indicated by the late peak in serum antibodies [4].

The observation that both serum and copro-antibodies are more effective orally than subcutaneously also supports our conception as to the pathomechanism of shigella infection. By oral administration presumably a higher antibody concentration can be attained in the intestines, at the very site where shigellae penetrate the epithelium and invade the intestinal lymphatic system. Concentrated antibodies may exert a more effective protection against a local infection such as dysentery.

As demonstrated in previous experiments, in order to obtain an effective immunity, the animals should be given large doses of vaccines [2]. The necessity for large doses is even more definite in passive immunization. The favourable protective effect of serum given after infection is also striking. Shigellae of course multiply freely in the intestine of mice lacking the normal coli flora due to streptomycin treatment. In the course of passive immunization, when the local antibody level decreases considerably, surviving shigellae may multiply again and penetrate through the epithelium. It may be supposed that prolonged administration of serum inhibits this phenomenon.

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PROPERTIES OF PENICILLIN ACYLASE ISOLATED FROM ESCHERICHIA COLI

By

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Summary. Penicillin acylase, an enzyme splitting and synthetizing penicillin-G, has been extracted by the usual methods (ammonium sulphate fractionation, calcium phosphate gel adsorption and DEAE cellulose chromatography) from *E. coli*, and purified fortyfold. With this purified enzyme preparation the kinetic data of splitting and synthetizing of penicillin-G were determined, and the substrate specificity was studied. The purified enzyme was found to be capable of splitting stereospecifically and at a high rate the derivatives of N-phenyl-acetyl-amino acids.

These experimental results suggest that the enzyme has a regulatory role in the trans-acylation process of cell metabolism. At the same time, the inhibitory experiments explain the effect of phenyl- and phenoxyacetic acid derivatives upon enzyme production; these acids cause derepression by inhibition of enzyme activity.

SAKAGUCHI and MURAO [1, 2] were the first to show splitting of penicillin-G by penicillin amidase. Ten years later ROLINSON *et al.* [3] demonstrated the practical importance of this enzymic reaction, in other words, that 6-aminopenicillanic acid, formed through the hydrolysis of penicillin-G, is an elementary starting substance of several semisynthetic penicillins. In view of the practical value of this recognition, several research groups have dealt with the production and properties of penicillin amidase [4—11]; it has been isolated from various microorganisms [12—14], and its substrate specificity has been discussed in detail [15—18].

In a previous communication a detailed report has been given about the production of penicillin acylase by *E. coli* [19]. It was demonstrated that enzyme production, stimulation, repression and derepression were strongly dependent on the temperature of cultivation. In the studies to be presented the properties of the purified enzyme were studied with special reference to its substrate specificity, in the hope that in the possession of these results it will be possible to explain the effect of various inducers upon enzyme production and to draw conclusions concerning the metabolic role of the enzyme.

Materials and methods

Microorganism. The organism employed was *E. coli* strain Ny. I/3, isolated by L. NYIRI.

Cultivation. Screening of cultures from penicillin acylase activity was carried out as follows. The medium was composed of 1.0 per cent Bacto casamine acids (Difco) and 0.1 per cent phenylacetic acid. Before sterilization the pH was adjusted to 7.0. The media were steri-

lized at 121° C for 30 minutes. Cultivation was maintained in a series of 10 litre glass fermentors with five litres of useful volume, at 24°C under 1 v/v/min aeration; they were inoculated with 100 ml of a 24-hour-old bouillon culture.

Enzyme assay. Enzyme activity was estimated at 40°C during a 20 min. reaction period. In the course of benzylpenicillin-splitting the reaction mixture contained 6 μM of penicillin-G in 0.05 M phosphate buffer pH 7.8. For estimation of enzyme synthesis 0.05 Mphosphate buffer pH 5.7 was used. The reaction mixture contained 6 μM 6-aminopenicillanic acid and 60 μM phenylacetic acid or some other side chain precursor. The reaction was stopped by adding ten volumes of absolute alcohol and cooling in ice. The pH was maintained at the given values ($\pm$ 0.1 pH) by the addition of alkali or acid. The various penicillin products were estimated biologically by the agar diffusion method. The 6-aminopenicillanic content was assayed biolog-

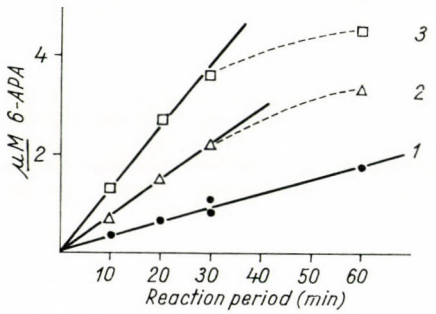

Fig. 1. Effect of protein concentration on reaction rate. One ml of the mixture contained 6 μM penicillin-G and various amounts of protein in 0.05 M phosphate buffer, pH 7.8; 1 : 0.3 mg/ml protein; 2 : 0.6 mg/ml protein; 3 : 1.2 mg/ml protein

ically after previous acylation by phenylacetyl chloride. The unit of specific activity was defined as 1 μM substrate split or 1 μM end product formed, per mg protein per hour.

Owing to the reversibility of the reaction, the formed product strongly inhibited the reaction rate. Consequently, the progress curve of enzyme reaction in time considerably deviated from the straight line at prolonged incubation times (Fig. 1). To eliminate this source of error, enzyme activities were estimated at short intervals and with protein concentrations causing ten substrate concentration to change with at most 25 to 30 per cent.

Low substrate concentrations (6 μM) were applied, to ensure favourable conditions for biological estimation.

Paper electrophoresis. Purified protein fractions were run at 180 V for 24 hours on Macheray-Nagel No 214 filter paper impregnated with veronal buffer (0.05 ionic-strenght, pH 8.6) in a "Labor" electrophoretic apparatus. For development, acidic fuchsin was applied. Chromatograms were bioautographed for identification of active protein spots by immersing paper strips equal in size to the electrophoresis strips into a solution containing 0.05 unit/ml of benzylpenicillin in 0.05 μM phosphate buffer pH 7.0; the strips were then dried at room temperature and stretched onto a wooden frame placed into a flat glass trough kept at 40°C and 100 per cent relative humidity. Then the electropherograms were placed onto the strips impregnated with penicillin and were evenly sprayed with 0.05 μM phosphate buffer pH 7.0. After incubation for two hours the strips were laid onto agar plates infected with *B. subtilis*. After 30 minutes the strips were removed, and the plate was kept at 37°C for 18 hours. The growth of bacteria clearly indicated the site of the enzyme.

Determination of protein content was carried out according to LOWRY *et al.* [22].

Materials. Commercial preparations of pure or analytical grade were used.

Purification of enzyme. The enzyme was purified by the usual procedures [4, 7. 12, 13, 14], in the following manner. After 22 hours fermentation broths were separated in a Sharples-centrifuge and washed twice with 31 of 0.03 M phosphate buffer pH 7.0 by centrifuging at 3.000 r.p.m. Then the cells were resuspended in 80 ml phosphate buffer (30—40 mg/ml) and sonicated for 8 min in a MSE magnetostrictic disintegrate (20.000 kc 50 W) cooled by ice water. The remaining cell debris was removed by centrifuging at 16.000 g, the cell-free enzyme solution

thus obtained was adjusted to 150 ml with the above buffer; it represented the starting crude extract.

To the crude extract 40 g of finely powdered ammonium sulphate was added. The pH was adjusted to 6.5, and after standing and occasional shaking for one hour in a refrigerator the precipitate was removed by centrifuging at 16.000 g. Five g of solid ammonium sulphate were added to the supernatant. After further two hours standing in the cold the precipitate was collected by centrifuging for 15 min at 16.000 *g*. The precipitate contained approximately 30 per cent of the activity (fraction B); the supernatant still contained high enzyme activity which could be precipitated by further addition of ammonium sulphate but the specific activity of this fraction was lower than that of fraction B, and was therefore discarded.

Two fractions were compared by paper electrophoresis. In both cases the active fraction migrated at the same rate.

Fraction B was further purified as follows. The pH was adjusted to 7.0 with dilute sodium hydroxide solution. To obtain an ion-free fraction, the solution was percolated through a Sephadex G 25 column equilibrated with 0.01 *M* phosphate buffer pH 7.0. To this ion-free solution were added 10 ml of calcium phosphate gel (10 per cent dry weight). After centrifugation the precipitate was washed twice with 15 ml of distilled water and eluted by phosphate buffer pH 7.8 at increasing concentrations (0.01, 0.04, 0.1, 0.5 *M)*. The major part of the activity was found in the 0.1 *M* eluate which usually contained 15 to 25 per cent of the initial enzyme activity. Specific activity was about 10 to 14 units, which as compared with the starting activity meant a 20 to 25 fold purification. In the experiments all measurements were carried out with enzyme purified as described above.

Chromatography of ion-free fraction B on DEAE cellulose column resulted in an enzyme solution of higher specific activity. After treatment with Sephadex G 25 the fraction was passed through a Whatman DEAE cellulose column (1.3 × 23) equipped with a cooled jacket. Elution was performed at increasing buffer concentrations. The mixer contained 65 ml of 0.001 *M* phosphate buffer pH 7.8; the container, 0.05 *M* phosphate buffer pH 8.0. Fractions of 3 ml were collected, 16 mg protein was applied to the column. Fractions 35 and 36 exhibited the highest activity, containing 10 per cent of the total starting activity. The specific synthetizing activity thus obtained was approximately 25 to 30 units.

Table I

Purification of penicillin acylase

	Specific activity of splitting	Total activity unit	Specific activity of synthesis	Total protein, mg	Production, per cent
Crude extract	0.6	540	0.08	900	—
Fraction B	2.1	243	0.32	120	45
0.1 *M* eluate	13.1	97	1.60	7.4	18
Fraction DEAE 35	29	29	3.50	1.02	10
36	23	24	3.55	1.05	

Results

Stability of the enzyme. After purification with phosphate buffer (pH 5.0 and 8.0) the enzyme was kept for two hours in a water-bath at various temperatures. Subsequently, the pH of the enzyme solutions was adjusted to 7.0 and 5.2, respectively, and the activity of samples was determined. In Table II the remaining activity is indicated. Values obtained without storage were considered 100 per cent. It is obvious from the data that the enzyme is more sensitive

at alkaline than acidic pH and its splitting and synthetizing activity decrease with the decrease of the pH.

Table II

Temperature sensitivity of penicillin acylase at alkaline and acid pH

Temperature of storage	Remaining activity			
	Storage at pH 5.0		Storage at pH 8.0	
	splitting	synthetizing	splitting	synthetizing
	activity, per cent		activity, per cent	
0°C	92	95	92	84
30°C	82	89	78	76
40°C	72	70	66	95
55°C	52	48	30	20

Protein concentration during heat treatment was 4.2 mg/ml.

The effect of temperature on the velocity of the reaction is seen in Figs. 2 and 3. The maximum splitting and synthetizing rate was achieved at 42°C. Above this temperature the rate decreased considerably. The decrease cannot

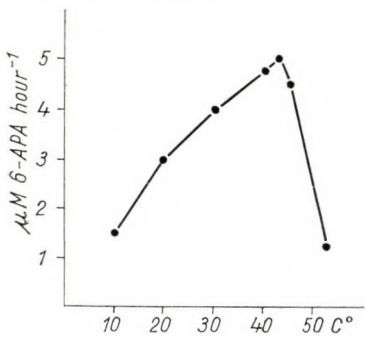

Fig. 2. Effect of temperature on splitting of penicillin-G. One ml of reaction mixture contained 6 μM benzyl-penicillin, and 0.4 mg protein in 0.05 M phosphate buffer, pH 7.8

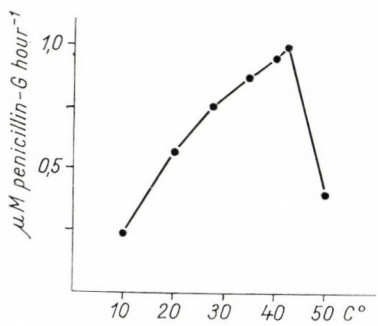

Fig. 3. Effect of temperature on penicillin G synthesis. One ml of the reaction mixture contained 6 μM 6-aminopenicillanic acid, 60 μM phenylacetic acid, and 1.1 mg protein in 0.05 M phosphate buffer, pH 5.2

be explained by the temperature sensitivity of the enzyme alone. Above 42°C the stability of penicillin-G and 6-aminopenicillanic acid rapidly decreases and this influences the values obtained which cannot be corrected by spontaneous breakdown curves, due to changes in concentration during the reaction. At 40°C, the temperature adopted for enzyme activity measurements, the break-

down of penicillin was about 10 per cent, thus within the limits of error of enzyme activity determination.

As Fig. 4 shows pH 8.0 was found to be the optimum for splitting. At higher values a considerable decrease in splitting activity occurred. This can be attributed only in part to the properties of the enzyme, since above pH 8.0 a significant decrease in the stability of penicillin-G was also observed. Synthesis was optimal between pH 4.5 and 5.0. (Fig. 5) Above this range synthesis decreased rapidly, to stop completely at pH 7.0. Below pH 4.5 the estimation of synthetizing activity was uncertain owing to the poor solubility of 6-amino-

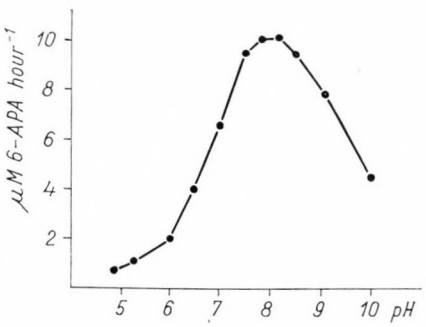

Fig. 4. pH dependence of penicillin-G splitting. One ml of the reaction mixture contained 6 μM penicillin-G and 2—0.8 mg protein in 0.05 M phosphate buffer

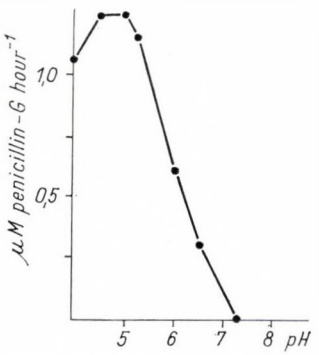

Fig. 5. pH dependence of synthetizing of penicillin-G. One ml of the reaction mixture contained 6 μM 6-aminopenicillanic acid, 60 μM phenylacetic acid, and 1 mg protein in 0.05 M phosphate buffer

penicillanic acid the iso-electric point of which is at pH 4.3. In the measurement of penicillin synthesis (as given under Methods) the concentration of phenylacetic acid was raised to one order of magnitude above that of 6-aminopenicillanic acid, because the equilibrium of the reaction shifts towards splitting even in the acid pH range.

This is proved by the equilibrium constant at pH 5.2. In the composition of the reaction mixture no measurable change could be detected after several hours of incubation when the components had been applied at the following concentrations: 6-aminopenicillanic acid, 4.2 μM; penicillin-G, 0.54 μM; phenylacetic acid, 74 μM. According to the above data the equilibrium constant of the reaction is

$$K = \frac{[4.2]\ [74]}{[0.54]} = 575$$

When the concentration of the above components was decreased to one half or raised twofold, no change in equilibrium could be observed.

We examined the effect of the concentration of phenylacetic acid on the synthesis and splitting of penicillin-G at pH 5.2, favourable for enzyme synthesis (Figs 6 and 7). On increasing the concentration of phenylacetic acid the rate of splitting decreased and the rate of synthesis increased. The inhibitory effect of phenylacetic acid on the splitting of penicillin-G could be observed also at higher pH values, but at alkaline pH the rate of inhibition decreased.

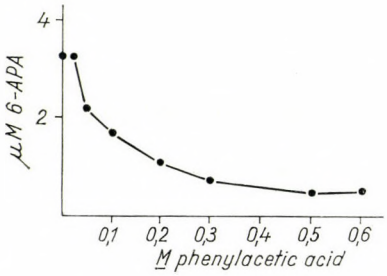

Fig. 6. Effect of phenylacetic acid concentration on splitting of penicillin-G at pH 5.2. One ml of the reaction mixture contained 6 μM benzylpenicillin, 2.1 mg protein, phenylacetic acid at various concentrations in 0.05 M phosphate buffer pH 5.2

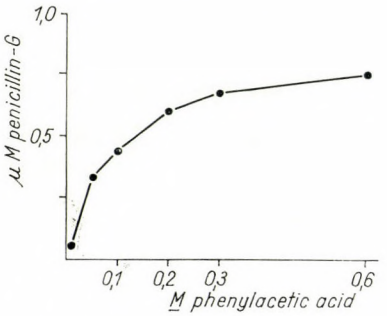

Fig. 7. Effect of phenylacetic acid concentration on the synthesis of penicillin-G. One ml of the reaction mixture contained 6 μM 6-aminopenicillanic acid, 2.1 mg protein, and phenylacetic acid at various concentrations in 0.05 M phosphate buffer, pH 5.2

As to the effect of various acylating agents (phenylacetic acid, phenoxyacetic acid, and p-methoxy-phenylacetic acid) on the splitting of penicillin-G, while the first two compounds inhibited splitting almost to the same extent, the inhibitory effect of p-methoxy-phenylacetic acid was considerably weaker (Fig. 8).

If the rate of synthesis was measured in the presence of the above compounds (Table III), phenoxyacetic acid inhibited penicillin-G synthesis and formed 100-times less penicillin within the same period than phenylacetic acid. The p-methoxy-phenylacetic acid incorporated slower than phenylacetic acid but considerably faster than phenoxyacetic acid.

Our results agreed well with the findings of KAUFMANN [16]. If instead of phenylacetic acid N-phenyl-acetylamino acid or phenylacetylthioglycolic acid was used, the rate of synthesis markedly increased. The enzyme is more active in the transacylation reactions. If instead of free phenylacetic acid, phenylacetyl-glycine was applied, a fivefold increase in the rate of reaction occurred.

The synthesis by this transacylase is stereospecific. In the presence of phenylacetyl-L-alanine the amount of penicillin formed was 15 times more than in the presence of phenylacetyl-D-alanine (Table III).

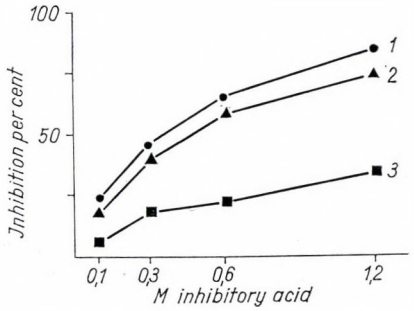

Fig. 8. Inhibition of penicillin-G by various acetic acid homologoues. One ml of the reaction mixture contained 60 μM penicillin-G, 1.8 mg protein, and inhibitory acids at various concentrations in 0.1 M phosphate buffer, pH 8.0. 1 : phenylacetic acid; 2 : phenoxyacetic acid; 3 : p-methoxyphenylacetic acid

Table III

Acylation of 6-aminopenicillanic acid by various acids

Acid added to the reaction mixture	Amount of penicillin measurable after 1 hr reaction period
60 μM phenylacetic acid	300 μg benzylpenicillin
60 μM phenoxyacetic acid	3 μg phenoxymethylpenicillin
60 μM phenylacetic acid + 60 μM phenoxyacetic acid	90 μg benzylpenicillin
60 μM p-methoxyphenylacetic acid	79 μg p-methoxy benzylpenicillin
15 μM N-phenylacetyl glycine	1600 μg benzylpenicillin
30 μM phenylacetyl thioglycolic acid	1200 μg benzylpenicillin
15 μM N-phenylacetyl-L-alanine	1550 μg benzylpenicillin
15 μM N-phenylacetyl-D-alanine	100 μg benzylpenicillin

The reaction mixture contained 1.2 mg protein, 6 μM 6-aminopenicillanic acid, and various acyl side-chain acids per ml, in 0.05 M phosphate buffer pH 5.2.

Results for the rate of splitting of various penicillin derivatives at pH 7.8 are summarized in Table IV. The splitting rate was almost the same with benzylpenicillin, phenoxymethylpenicillin, and ampicillin, but oxacillin was not split. Results with penicillin-V were remarkable if compared with the data in Fig. 8 and Table III. While splitting of penicillin-V did not differ significantly

from that of penicillin-G, a marked difference was noted in the rate of synthesis.

Finally, since our aim has been to investigate the phenomena concerning acylase inducibility, we determined under conditions described in a previous paper [19] the effect upon enzyme induction of the phenylacetic acid derivatives synthetized by us (Table V). Induction by phenylacetic acid, phenylacetylglycine, and phenylacetyl-L-alanine were nearly similar, while phenylacetyl-D-alanine was ineffective in this respect.

Table IV

Splitting of various penicillins of pH 7.8

Substrate	Formation of 6-aminopenicillanic acid during 20 min
Penicillin-G	3.1 μM
Penicillin-V	1.9 μM
Ampicillin	2.1 μM
Oxacillin	0.0 μM

The reaction mixture contained 0.5 mg protein and 6 μM various penicillins in 0.05 M phosphate buffer pH 7.8.

Table V

Effect on acylase synthesis of addition to the medium of various phenylacetic acid derivative

Inductor (0.02 M)	Acylase activity
—	1.1
Phenylacetic acid	8.2
Phenylacetyl glycine	8.1
Phenylacetyl-L-alanine	7.6
Phenylacetyl-D-alanine	1.2

Discussion

The name of the enzyme in question has formed the subject of discussions. The name penicillin amidase proposed by the first describers [1] was favoured by several authors. Others preferred to call the enzyme penicillin acylase [15]. Recent reports and our own data prove that both names are incorrect [16, 17], since the enzyme does not seem to be responsible for the intracellular cleavage of penicillin but catalizes certain transacylation processes.

Owing to the similarity in chemical structure, the enzyme is well suited for the industrial preparation of 6-aminopenicillanic acid.

Numerous research groups have dealt with the purification of penicillin acylase. BATCHELOR *et al.* [12] isolated extracellular acylase from *Streptomyces lavendulae*. They achieved a fivefold purification by precipitation with ammonium sulphate and fractionation with acetone. The enzyme split penicillin-V. In contrast to our results, these authors found that phenoxy-acetic acid added to the reaction mixture does not affect the reaction rate, while 6-aminopenicillanic acid considerably decreases it.

MURAO *et al.* [7] isolated and purified the extracellular acylase of *B. megaterium* in a similar way. CLARIDGE *et al.* [4] isolated intracellular acylase from

Alcaligenes faecalis. They observed a quantitative difference in activity between toluene-treated cells and the cell-free extract obtained by ultrasonic treatment. The rate of enzymic splitting of penicillin-G was the same in both cases, while the splitting of penicillin-V decreased after disintegration. In our experiments with the employed organism such a phenomenon was not observed.

BORKAR *et al.* [13] isolated an intracellular penicillin acylase from *E. coli*. A twentifivefold purification could be attained when the enzyme was fractionated with ammonium sulphate and chromatographed on DEAE cellulose column.

WALDSCHMIDT-LEITZ *et al.* [14] isolated from the acetone dried powder of *Fusarium semitectum* mycelia an intracellular enzyme capable of splitting penicillin-V. They succeeded in attaining a threehundredfold purification. Measuring the molecular weight of the enzyme by means of ultracentrifugation, it was found to be 65.000. The enzyme proved to possess two Zn atoms per molecule. In our enzyme the activating effect of metal ions could not be demonstrated and its activity was not decreased by 139 μg/ml of 8-hydroxychinoline.

As to the specificity of the active centre of the enzyme, the most important point is the specificity towards L-amino acids, phenylacetic acid and phenoxyacetic acid. In fungi and Actinomycetes penicillin-V, while in bacteria penicillin-G is split by the enzyme at the highest rate. The enzyme readily hydrolysing penicillin-G is entirely intracellular; it hydrolyses phenylacetyl glycine even more readily but has no effect upon N-acetyl glycine [17].

The effect of phenoxyacetic acid on enzyme activity was specially remarkable. Penicillin-V was split at a sufficient rate but synthetized only in traces. The situation was rendered even more complicated by the finding that phenoxyacetic acid, similarly to phenylacetic acid, inhibited both the splitting and the synthesis of benzylpenicillin. Thus it is bound to the active centre but, due to its steric difference, it inhibits the activity of the enzyme. On the other hand, if phenylacetic acid is introduced in the form of phenoxyacetyl glycine, or thioglycolic acid thioester is added to the reaction mixture [23, 24], the process of transacylation is more enhanced than direct acylation.

The configuration of the acylated amino acid is a substantial criterion of activity, the enzyme being specific for acylated L-amino acids only, thus N-phenylacetyl-D-alanine was not affected by the enzyme. The arrangement in the penicillin molecule of the two carbon atoms at issue exactly corresponds to the configuration of L-amino acids. The surface of enzyme is presumably specific only for these two carbon atoms, the α- and carbonyl carbon atoms, and activity differences due to differences in the size of the acid molecule might also be diminished, *e.g.* in the case of phenoxyacetic acid one oxygen atom which is longer than phenylacetic acid. The presence of the carbonyl atom is essential because the enzyme is unable to incorporate phenylacetic acid esterified by mercaptoethanol. It appears that the enzyme is not so sensitive to sub-

stituents on the phenyl group, since it is capable of directly incorporating p-methoxyphenylacetic acid. On the other hand, sensitivity is increased towards phenylacetic acid derivatives substituted at the α-carbon atom. The incorporation of phenyl glycine for example proceeds poorly while it is more readily incorporated from glycolic acid-thioester.

The practical use of this observation is hindered by the poor water-solubility of the thioester.

Concerning the inducibility of the enzyme, we have failed to detect differences between phenylacetic acid, phenylacetyl thioglycolic acid, or N-phenylacetyl glycine and N-phenylacetyl-L-alanine. This is only natural since the acylase being present rapidly cleaved the added compound. N-phenylacetyl-D-alanine was of course ineffective not only as substrate but also as inducer.

No substantial difference in inductive effect could be demonstrated between phenylacetic acid and phenoxyacetic acid [19]. This shows that instead of phenylacetic acid, phenoxacetic acid can be bound to the surface essential for enzyme induction. Inhibition of enzyme synthesis was similar with phenylacetic and phenoxyacetic acid. P-methoxy-phenyl acetic acid, which is a considerably weaker inhibitor of the enzyme, is weaker also as inducer.

It may be concluded that under certain conditions acylase might have an important role in cell metabolism especially on carbohydrate-free media with a high aminonitrogen content. On media containing carbohydrates, especially at higher temperatures (38—40°C), the role of the enzyme is less important, and it may even cause repression [19]. If to the medium phenylacetic acid or some other inducer is added, this is bound to the active centre of the enzyme and inhibits its normal function. This inhibition results in a disturbance of cell metabolism, and production of the enzyme is derepressed.

Acknowledgement. The author is indebted to Dr. J. Bérdy for preparation of the N-phenylacetyl derivatives.

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ACTA MICROBIOLOGICA

ТОМ XII

РЕЗЮМЕ

ANTIGENIC STRUCTURE OF SAPROPHYTIC MYCOBACTERIA

F. Gimpl

АНТИГЕННАЯ СТРУКТУРА САПРОФИТНЫХ МИКОБАКТЕРИЙ

Ф. Гимпл

Автором изучена антигенная структура сапрофитных микобактерий путем иммунодиффузии и иммуноэлектрофореза. На основании сравнения антигенной структуры исследованные микобактерии были распределены в 3 иммунологические группы.

I группа: *M. smegmatis*, *M. friburgensis*, *M. butyricum;* II группа: *M. phlei;* III группа: *M. pellegrino.* Найдена основная разница между антигенными структурами сапрофитных штаммов микобактерий и *M. tuberculosis*, с одной стороны, а с другой — между антигенной структурой двух фотохромогенных штаммов.

SALICIN-FERMENTING VARIANT OF SHIGELLA FLEXNERI SEROTYPE 2A

L. Kerekes

СЕРОТИП 2А SHIGELLA FLEXNERI, ФЕРМЕНТИРУЮЩИЙ САЛИЦИН

Л. Керекеш

Автор знакомит со штаммом *Shigella flexneri* 2a, который в различных биотиках параллельных субкультур представляет целый ряд переходных форм, начиная от сорбит-положительного биоварианта *Sh. flexneri* 2a, через особи, разлагающие рамнозу, до салицино-положительного варианта, считаемого промежуточным членом в группе *Shigella* — *E. coli*. Штамм был изолирован от больного, имевшего дизентерийные явления, и после прививки в глаз морской свинки вызывал кератоконъюнктивит шигеллоза.

OBSERVATIONS ON BIOCHEMICAL CHANGES IN IRRADIATED SPORES OF BACILLUS CEREUS

J. Farkas, I. Kiss

ИССЛЕДОВАНИЕ БИОХИМИЧЕСКИХ ИЗМЕНЕНИЙ В ОБЛУЧЕННЫХ СПОРАХ BACILLUS CEREUS

Й. Фаркаш, И. Кишш

После облучения концентрированных водных взвесей спор Bacillus cereus в спокойном состоянии изучено изменение содержания дипиколиновой кислоты спор. Спектрофотометрическими и хроматографическими методами изучены вещества, изливающиеся из бактериальных клеток на действие летальных лучевых доз.

Установлено, что обнаруживаемое в спорах в спокойном состоянии «псевдо-прорастание», возникающее под воздействием больших лучевых доз, может зависеть от увеличения проницаемости мембраны цитоплазмы. Повидимому, для повреждения мембраны цитоплазмы необходимы значительно более высокие дозы облучения, чем для дезорганизации процессов, вызывающих деление клеток.

На действие облучения из спор изливаются в окружающую среду такие вещества, которыми характеризуется и экссудат физиологического прорастания.

INDUCED LYSOGENESIS OF MYCOBACTERIA

E. Vandra, Á. Takáts

ИНДУЦИРОВАННЫЙ ЛИЗОГЕНЕЗ У МИКОБАКТЕРИЙ

Е. Вандра, А. Такач

С помощью литических фагов сапрофитные микобактерии были искусственно превращены в лизогенные штаммы. Из них изучены лизогенные комбинации *M. friburgensis* и *M. rabinowitsch* и их гомологичных фагов.

В отношении морфологии колоний и биохимического поведения лизогенные штаммы не отличались от исходных штаммов.

Лизогенное состояние прекратило литическое действие гомологичного и родственного фага. Чувствительность лизогенных культур к другим фагам или едва изменялась, или совершенно не изменялась.

Упомянутые 4 лизогенных штамма ультрафиолетовым облучением индуцировать не могли.

ADENOVIRUS TYPE 7 OUTBREAK IN A KINDERGARTEN

M. Tóth, P. Osváth

ВСПЫШКА ЗАБОЛЕВАНИЙ, ВЫЗВАННАЯ АДЕНОВИРУСОМ ТИПА 7, В ДЕТСКОМ САДУ

М. Тот, П. Ошват

Авторы знакомят со вспышкой заболеваний дыхательных путей, протекавшей в детском саду одного из периферических районов Будапешта в январе 1964 года, где среди 36 детей заболели 26 (72%). Попытки изолировать вирус из выделений носа и горла проводились в 22 случаях, в результате чего удалось от 14 больных изолировать аденовирус типа 7. У одного больного из одновременно взятых выделений носа и горла были изолированы 7-ой (выделения носа) и 3-ий (выделения горла) типы аденовируса. Изучены 19 парных сывороток методом связывания комплемента и торможения гемагглютинации с 3 и 7 типами аденовируса. Реакция торможения гемагглютинации оказалась более чувствительной, с её помощью удалось подтвердить в 17 случаях связь заболевания с аденовирусом типа 7. При применении реакции связывания комплемента получили положительный результат в 9 случаях.

STUDIES ON THE FERMENTATION OF STEREUM PURPUREUM

Z. Cseri

ИЗУЧЕНИЕ ФЕРМЕНТАЦИИ STEREUM PURPUREUM

З. Чери

Stereum purpureum, с точки зрения культивируемости, соответствует требованиям ферментационной промышленности. При применении соответствующей технологии можно производить культуру гомогенной дисперсности. Образование комочков зависит от состава питательной среды и от значения ее pH. Образование пигмента зависит от последовательности применяемых питательных сред в течение пассажей и от pH питательной среды.

AMYLASE ACTIVITY OF SUBMERGED STEREUM PURPUREUM CULTURES

Z. Cseri

АМИЛАЗА-АКТИВНОСТЬ ПОГРУЖЕННОЙ КУЛЬТУРЫ STEREUM PURPUREUM

З. Чери

При изучении амилаза-активности погруженной, аэрированной, гомогенной культуры *Stereum purpureum* установлено:

1. Количество крахмала в питательной среде, содержащей крахмал и сою, а также pH при стерилизации изменяются.
2. Гомогенной погруженной культурой *Stereum purpureum* быстро разлагается крахмал питательной среды.
3. Активность амилазы Stereum, по мере её концентрации, задерживается продуктами распада, возникающими при стерилизации питательной среды.
4. Для изготовления ферментного сока с высокой амилаза-активностью наиболее пригодной является питательная среда, содержащая крахмал и сою, pH которой установлен до стерилизации на 4,0.
5. Ферментный сок Stereum, вероятно, содержит и энзим, разлагающий глюкозидные связи 1,6.

EFFECT OF pH ON THE GROWTH IN SUBMERGED CULTURE OF SOME WOOD-ROTTING FUNGI

Z. Cseri

ВЛИЯНИЕ pH НА РОСТ НЕКОТОРЫХ ГРИБОВ, ПОВРЕЖДАЮЩИХ ДЕРЕВЬЯ, В ПОГРУЖЕННОЙ КУЛЬТУРЕ

З. Чери

Установлено, что *Stereum purpureum*, *Trametes versicolor* и *Phellinus igniarius* в погруженной аэрированной, гомогенной культуре пользуются глюкозой в качестве источника энергии. Эти виды продуктировали больше мицелиев на питательной среде, содержащей сою и крахмал, чем при использовании питательной среды с экстрактом сои и глюкозой. Оптимальное значение pH при применении питательной среды, содержащей сою и глюкозу, в зависимости от вида различно. Интенсивность роста у *Trametes versicolor* и *Phellinus igniarius* показывает тенденцию к снижению в направлении более кислых значений pH. Кривая роста Stereum purpureum отличается от этого, потому что при значениях pH 7,0 и 5,0 она показывает очень слабый рост.

AMINO ACID SENSITIVITY OF STRAINS DERIVED FROM ESCHERICHIA COLI K_{12}

I. Gadó, I. Horváth

ЧУВСТВИТЕЛЬНОСТЬ К АМИНОКИСЛОТАМ ШТАММОВ, ПРОИСХОДЯЩИХ ИЗ ESCHERICHIA COLI K_{12}

И. Гадо, И. Хорват

Было показано и у штаммов стрингентов явление, подобное аминокислотному торможению, обнаруженному Alföldi и сотрудниками у ослабленных штаммов. Задержки эти отличались от валиновой задержки, созданной в результате влияния «feed-back». Лаг-фазу, вызванную «shift-down», можно было прекратить валином и изолейцином. Допустимо, что лейцином расстраивается депрессия энзимов, участвующих в синтезировании аминокислот, располагающих разветвлёнными углеродными цепочками.

TRANSDUCTION IN BACILLUS SUBTILIS

K. Csiszár, G. Ivánovics

ИССЛЕДОВАНИЕ ТРАНСДУКЦИИ BACILLUS SUBTILIS

К. Чисар, Г. Иванович

(1) Сравнительное изучение описанных до сих пор различных темперированных фагов **B. subtilis** показало, что они причисляемы к 2 группам. В качестве общего госта этих фагов штамм *B. subtilis* **NRS** 231 явился применяемым, так как он оказался одинаково чувствительным в отношении всех изученных темперированных фагов. Упомянутый штамм является и из-за определенных технических преимуществ чрезвычайно пригодным для оценки темперированных фагов **B. subtilis**.

(2) Между ауксотрофными штаммами **B. subtilis 168 ind**, сохраненными в различных лабораториях, обнаружены культуральные разницы. Популяция одного из этих штаммов состояла из бактерий, ассимилирующих и не ассимилирующих аммиак. Выяснилось, что способность ассимилировать аммиак трансдукцией может быть передана не ассимилирующим аммиак бактериям.

(3) Изученные штаммы **B. subtilis** высвобождали **mitomycin** C или же после у. ф. индукции начало, подобное **«bacteriocin»**. Это явление является, вероятно, аналогичным дефективным образованиям фага **PBSX**, описанным **Seaman** и сотрудниками. На основании **«bacteriocin»** изученные штаммы можно было разделить на 2 группы.

(4) Изолированные авторами темперированные бактериофаги, повидимому, были идентичными с фагом **PBSI**. На основании антигенной структуры и **«host range»** от них отчетливо можно было отличать фаг **SPIO**.

(5) Отдельные лизаты фагов типа **PBSI** трансдуцировали с частотой величины 10^{-6}. Возникали и значительно более или менее эффективные популяции фагов из отдельных лизогенных трансдуцентов. На основании этого могли получить фаговые вещества, трансдуцирующие в величине 10^{-4}.

(6) На частоту трансдукции, кроме фагового вещества, влияло и физиологическое состояние бактерий.

(7) Авторы изучали на **his**$^-$ производных штамма 168 **ind**$^-$ трансдукцию, связанную с **ind** и **his locus**-ами.

β-GALACTOSIDASE ACTIVITY OF SACCHAROMYCES FRAGILIS II. MODE OF ACTION

G. Szabó, J. Rózsa

АКТИВНОСТЬ β-ГАЛАКТОЗИДАЗЫ SACCHAROMYCES FRAGILIS II. ИЗУЧЕНИЕ МЕХАНИЗМА ДЕЙСТВИЯ

Г. Сабо, Й. Рожа

Установлено, что обработка клеток **Saccharomyces fragilis** пирофосфатом натрия (**Na** -P -P) увеличивает активность их β-галактозидазы. Активность энзима увеличивалась и другими веществами, оказывающими влияние на обмен веществ, а также определенными энзимными ядами, как например, цианистым калием, фтористым натрием, азидом натрия, уранилнитратом. При одновременном применении **Na** -P -P и азида натрия воздействие на активность энзима суммировалось. Увеличение активности энзима, появляющееся после обработки **Na** -P -P, невозможно было задержать 20 различными, подавляющими энзим средствами. С этой точки зрения эффективным оказался только фенил-меркуриацетат.

Найдена корреляция между активностью β-галактозидазы и количеством растворяемого и экстрагируемого при температуре 0° C фосфата. Воздействия, которые повышали количество экстрагируемых водой фосфатов, увеличивали и активность β-галактозидазы.

POSSIBLE CAUSES OF LEUCINE INHIBITION IN ESCHERICHIA COLI K_{12} λ-28

I. Horváth, I. Gadó

ВОЗМОЖНЫЕ ПРИЧИНЫ ЧУВСТВИТЕЛЬНОСТИ К ЛЕЙЦИНУ У ШТАММА ESCHERICHIA COLI K_{12} λ—28

И. Хорват, И. Гадо

Лейцин, путем удлинения лаг-фазы, задерживает рост *E. coli* K_{12} λ — 28. Основами пурина и пиримидина усиливается упомянутое влияние, и наоборот, антагонисты этих соединений — уменьшают. Если промытые клетки при наличии лейцина обрабатывать хлорамфениколом, чувствительность клеток к лейцину повышается. Экспериментальные данные указывают на то, что при возникновении лейцин-торможения играет роль обмен веществ рибонуклеиновой кислоты.

A NEW COMPONENT FROM THE CELL WALL OF STREPTOMYCES GRISEUS. I. THE ROLE OF STREPTOMYCIN IN THE LIFE OF STREPTOMYCES GRISEUS

G. Szabó, Gy. Barabás, T. Vályi-Nagy, Zs. Magyar

НОВЫЙ КОМПОНЕНТ, ПОЛУЧЕННЫЙ ИЗ СТЕНКИ КЛЕТКИ STREPTOMYCES GRISEUS

I. РОЛЬ СТРЕПТОМИЦИНА В ЖИЗНЕДЕЯТЕЛЬНОСТИ STREPTOMYCES GRISEUS

Г. Сабо, Дь. Барабаш, Т. Вайи-Надь, Ж. Мадяр

Изучались мицели штамма-мутанта *Streptomyces griseus*, который потерял способность выделять Streptomycin. Изучены препараты из стенки клеток для установления того, можно ли обнаружить в них составные части молекулы стрептомицина. Удалось изолировать стрептидин, и из этого сделали вывод, что в качестве составной части клеточной стенки таких штаммов фигурирует стрептомицин.

ISOLATION OF CYTOMEGALOVIRUS AND INCIDENCE OF COMPLEMENT-FIXING ANTIBODIES AGAINST CYTOMEGALOVIRUS IN DIFFERENT AGE GROUPS

L. Váczi, É. Gönczöl, F. Lehel, L. Géder

ИССЛЕДОВАНИЯ ПО ВЫЯВЛЕНИЮ ВИРУСА ЦИТОМЕГАЛИИ И В ОТНОШЕНИИ ЧАСТОТЫ ВСТРЕЧАЕМОСТИ КОМПЛЕМЕНТСВЯЗЫВАЮЩИХ АНТИТЕЛ ПРОТИВ ВИРУСА ЦИТОМЕГАЛИИ В РАЗЛИЧНЫХ ВОЗРАСТНЫХ ГРУППАХ

Л. Ваци, Э. Гёнцёл, Ф. Лехел, Л. Гедер

(1) Исследуя мочу 16 — отчасти бессимптомных недоношенных, отчасти грудных детей, страдавших клинически явной формой цитомегалии или интерстициальным воспалением легких — изолировали в 4 случаях цитопатогенный агент.

Вирусы, на основании свойств культивирования, ими вызванных цитопатогенных изменений и проб нейтрализации, оказались возбудителями цитомегалии. Авторы знакомят со свойствами штаммов.

(2) В крови лиц различных возрастных групп с течением времени комплементсвязывающие антитела против вируса цитомегалии могут быть выявлены во всё возрастающем количестве. Из исследованных 442 сывороток сыворотки 2—24-месячных детей в

18%, 4—14-летних в 28%, 14—30-летних в 35%, а сыворотки лиц старше 30 лет в 51% содержали комплементсвязывающие антитела против вируса цитомегалии в титре 1:4 или выше.

Титры комплементсвязывающих антител в сыворотке умственно отсталых детей были на том же уровне, как и в сыворотке здоровых детей соответствующих возрастных групп.

AETIOLOGY OF KERATOCONJUNCTIVITIS IN EPIDEMIC AND NON-EPIDEMIC PERIODS

Á. Jancsó, M. Simon

ИССЛЕДОВАНИЯ, КАСАЮЩИЕСЯ ЭТИОЛОГИИ ЗАБОЛЕВАНИЙ КЕРАТОКОНЪЮНКТИВИТОМ В ЭПИДЕМИЧЕСКОМ И МЕЖЭПИДЕМИЧЕСКОМ ПЕРИОДАХ

А. Янчо, М. Шимон

В течение эпидемии кератоконъюнктивита, протекавшей в Будапеште с декабря 1961 года по декабрь 1962 года, а также в последующем межэпидемическом периоде, продолжавшемся полтора года, беспрерывно проводились попытки изолирования аденовируса и серологические исследования в связи с заболеваниями, клинически считающимися кератоконъюнктивитом. Выделение вируса проводилось из 51 материала, взятого в эпидемическом периоде. Изолированы 1 штамм аденовируса третьего типа и 4 — восьмого типа. Результаты исследования проб крови больных (реакциями связывания комплемента и задержки гемагглютинации) показали, что во время эпидемии 63% слчуаев вызывались аденовирусом типа 8.

В этиологии спорадических случаев, по данным выделения вируса и серологических исследований, проведенных в межэпидемическом периоде в отношении 93 различных материалов, взятых от 81 больного, страдавшего эпидемическим кератоконъюнктивитом, играли роль и другие типы (3, 4, 7, 14, 16) аденовируса и вирус **Herpes simplex**. Заболевания кератоконъюнктивитом, обнаруженные в межэпидемический период, только в 15% случаев вызывались аденовирусом типа 8.

A NEW PHAGE TYPE OF STAPHYLOCOCCUS AUREUS ASSOCIATED WITH AN OUTBREAK OF PEMPHIGOID

É. Kende, T. Balló, E. Ferenczi

ИССЛЕДОВАНИЕ ШТАММА STAPHYLOCOCCUS AUREUS, ПРИНАДЛЕЖАЩЕГО К НОВОМУ ФАГОВОМУ ТИПУ И ВЫЗЫВАЮЩЕГО ЭПИДЕМИЮ ПЕМФИГОИДА

Е. Кенде, Т. Балло, Е. Ференци

Авторы сообщают о стафилококковом штамме, лизирующемся новым фагом. Новый фаг, который получился путем адаптации фага 42D к больничному, стандартными фагами не типируемому штамму, был назван ими 42 D/I. Распространение стафилококкового штамма с фаговым типом 42 D/I обнаружено в течение стафилококковой эпидемии, протекавшей в отделении новорожденных; наблюдения проводились в течение 41 недели. В этот период было проведено 5689 исследований относительно культивирования из носового отделяемого и пемфигоида, а также из кала новорожденных, из носового отделяемого и из молока матери, из носового отделяемого больничного персонала; стафилококки были выделены из 3088 материалов. Среди них фаговой тип 42 D/I был представлен в 44,7%. Стафилококковый штамм с упомянутым фаговым типом оказался в течение эпидемии опасным, патогенным штаммом, потому что он выделялся из 81,3% нагноений кожи новорожденных. Из носового отделяемого новорожденных, заболевших пемфигоидом, удалось выделить стафилококковый штамм фагового типа 42 D/I в 64,4% случаев, тогда как тот же штамм изолировался из носового отделяемого новорожденных, не имевших

пемфигоид, только в 39,2% случаев. Частота выделения опасного штамма в течение 5-месячного периода исследований не была одинакова, в этом отношении обнаружились большие колебания. Число стафилококковых нагноений было наименьшим, когда число выделителей 42 D/I снизилось тоже до минимума, независимо от количества выделителей стафилококка. Среди 1238 изученных штаммов 42 D/I оказались резистентными к пенициллину 99,8%, к стрептомицину 87,9%, к хлороциду 12,6%, к тетрациклину 21,3%, к неомицину 3,4% и к эритромицину 97,8%.

THE OCCURRENCE OF MICROSPORON COOKEI IN HUNGARY

J. Galgóczy

ВСТРЕЧАЕМОСТЬ MICROSPORON COOKEI В ВЕНГРИИ

Й. Галгоци

Автор показал встречаемость в Венгрии *Microsporon cookei* Ajello 1959 в пробах почвы.

EFFECT OF HEPARIN ON THE GROWTH OF THE HERPES GROUP OF VIRUSES

É. Horváth, G. Hadházy

ВЛИЯНИЕ ГЕПАРИНА НА РАЗМНОЖЕНИЕ ВИРУСОВ ГРУППЫ HERPES

Е. Хорват, Дь. Хадхази

Изучено влияние гепарина на размножение вирусов **Herpes varicellae, Cytomegalovirus** и **Herpes simplex** во вторичных человеческих фибробластных, **HeLa** и вторичных человеческих тиреоидных тканевых культурах. Добавление гепарина к тканевой культуре производилось — относительно момента инокуляции вируса — в различные сроки. Наибольшее снижение инфективного титра (**Herpesvirus**), или наиболее выраженная редукция очага (**Varicella, Citomegalovirus**) вызывались гепарином, когда он добавлялся к тканевой культуре одновременно с инокуляцией вируса. Величина подавления размножения вируса зависела от количества применяемого гепарина, а также от того, в какой тканевой культуре производилось исследование. В присутствии 10γ/мл гепарина инфективный титр вируса **Herpes simplex** на вторичных человеческих фибробластах на $10^{1.5}$, а на **HeLa** на $10^{3.4}$ оказался меньше, чем в контроле. По данным исследований, проведенных с вирусом цитомегалии, гепарином задерживается адсорбция вирионов.

RHODOTORULA ZSOLTII N. SP. A NEW SPECIES OF YEASTS, AND SOME NOTES ON THE TAXONOMY OF THE GENUS RHODOTORULA

J. Galgóczy, E. K. Novák

ОПИСАНИЕ НОВОГО ВИДА ГРИБА RHODOTORULA ZSOLTII N. SP. И НЕКОТОРЫЕ ЗАМЕЧАНИЯ К ТАКСОНОМИИ GENUS RHODOTORULA.

Й. Галгоци, Е. К. Новак

Авторы знакомят с новым видом гриба, который назвали *Rh. zsoltii n. sp.*. Он образует пигмент красного цвета, ассимилирует глюкозу, галактозу (слабо) и сахарозу (слабо), но не ассимилирует мальтозу, лактозу, раффинозу, нитрат и этиловый спирт. Гриб разлагает арбутин, но не образует вещества, подобного крахмалу.

ANTISTREPTOKINASE TITRE IN HEALTHY ADULTS

J. Böszörményi

ТИТР АНТИСТРЕПТОКИНАЗЫ У ЗДОРОВЫХ ВЗРОСЛЫХ

Й. Бёсёрмени

Автор изучил модифицированным методом титр антистрептокиназы у 400 здоровых доноров. В качестве сравнительного стандарта применялся им гамма-глобулин, равноценный стандарту антистрептолизина-0. Титры 96,25% обследованных распределились по правилам случайного разделения, однако 3,75% показали более низкие величины, чем это можно было ожидать. Средний титр всех обследованных оказался равным 115,6 единицы. На основании собственных исследований автор считает патологическими величины, превышающие 286 единиц. Средние титры по возрастным группам с течением времени снижались. Как общее среднее, так и среднее по возрастным группам, хорошо совпадали со средними титрами антистрептолизина-0 у доноров.

COMPARISON OF ANTISTREPTOKINASE AND ANTISTREPTOLYSIN-O TITRE IN HOSPITALIZED CHILDREN

J. Böszörményi, P. Baranyai

СРАВНИТЕЛЬНОЕ ИЗУЧЕНИЕ ТИТРОВ АНТИСТРЕПТОКИНАЗЫ И АНТИСТРЕПТОЛИЗИНА-О У БОЛЬНЫХ ДЕТЕЙ

Й. Бёсёрмени, П. Бараняи

Исследованы титры антистрептолизина-О и антистрептокиназы у 500 больных детей и из полученных данных сделан вывод, что реакция антистрептокиназы до некоторой степени более специфически показывает стрептококковую этиологию, чем реакция антистрептолизина-О. Параллельное проведение обоих реакций считается обоснованным, потому что в 26% случаев только одна реакция выявила стрептококковую инфекцию. Титры, получаемые реакцией антистрептокиназы, появлялись в более широкой зоне, чем титры антистрептолизина-О, однако применённым методом титрования граница нормальных и патологических величин обнаруживалась в обоих реакциях между 240 и 280 единицами.

STUDIES ON STAPHYLOCOCCUS AUREUS L FORMS BLOCKED AT DIFFERENT STAGES OF CELL WALL SYNTHESIS

M. Fodor, B. Tóth

ИЗУЧЕНИЕ ФОРМ L STAPHYLOCOCCUS AUREUS, ЗАТОРМОЖЕННОГО В РАЗЛИЧНЫХ СОСТОЯНИЯХ СИНТЕЗА КЛЕТОЧНОЙ СТЕНКИ

М. Фодор, Б. Тот

Авторы, путем обработки пенициллином, из штамма *Staphylococcus aureus* № 100 получили 4 различных, в жидких питательных средах хорошо культивируемых штамма L. 2 из штаммов L при наличии пенициллина продуцировали приблизительно в 5 раз больше по количеству нуклеотида, содержащего ацетиламинный сахар и растворяемого в кислоте, чем два другие штамма L. Накопленные нуклеотиды оказались уридин дифосфомураминкислотными пептидами. В питательной среде, не содержащей пенициллин, образование нуклеотидов **Park** стабильной формой L осталось на том же уровне, тогда как при наличии пенициллина синтез нуклеотидов **Park** у 2 штаммов L, образующих много нуклеотидов **Park**, сильно снизился.

STUDIES OF THE AEROBIC ENTERIC FLORA OF INFANTS

I. Némedi

ИССЛЕДОВАНИЕ АЭРОБНОЙ КИШЕЧНОЙ ФЛОРЫ ГРУДНЫХ ДЕТЕЙ

И. Немеди

При исследовании 6890 проб кала автором выявлено, что за исключением стафилококков встречаемость аэробных условно патогенных бактерий одинакова у здоровых и показывающих энтеральные симптомы грудных детей. При более подробной серологической и биохимической идентификации выделенных условно патогенных бактерий выяснилось, что внутри отдельных видов бактерий среди больных ни один тип не встречается чаще, чем среди лиц без всяких симптомов.

VIROLOGICAL INVESTIGATION OF HOSPITALIZED CASES OF PSEUDOCROUP AND ACUTE LARYNGOTRACHEOBRONCHITIS

M. Tóth, V. Major

ВИРОЛОГИЧЕСКОЕ ОБСЛЕДОВАНИЕ БОЛЬНЫХ, СТРАДАВШИХ ПСЕВДОКРУПОМ И ОСТРЫМ ЛАРИНГОТРАХЕОБРОНХИТОМ

М. Тот, В. Майор

С конца ноября 1963 г. до конца ноября 1964 г. проводились опыты по выделению вируса из глоточного отделяемого 423 грудных и маленьких детей, принятых в ларингологическое отделение с диагнозом: псевдокруп — острый ларинготрахеобронхит, а с 229 парными сыворотками были проведены различные серологические исследования.

Выделили всего 261 штамм вируса, среди них оказались вирусом параинфлюэнзы 3-го типа 34,1%, вирусом RS 23%, аденовирусом 17,6%, вирусом параинфлюэнзы 2-го типа 16,1%, положительным гемадсорбирующим агентом 5,7%, энтевирусом 1,9% и вырусом параинфлюэнзы 1-го типа 1,1%.

Уточнение этиологии удалось при одновременном применении выделения и серологических методов в 128 случаях, с помощью одного метода изолирования вируса — в 133 случаях и только серологическим путем — в 58 случаях.

INCIDENCE OF SV-40 VIRUS NEUTRALIZING ANTIBODIES IN SERA OF LABORATORY WORKERS

L. B. Horváth

ВСТРЕЧАЕМОСТЬ SV—40 ВИРУСНЕЙТРАЛИЗУЮЩИХ АНТИТЕЛ В КРОВИ РАБОТНИКОВ ЛАБОРАТОРИИ

Л. Б. Хорват

(1) Среди 30 лиц, имеющих отношение к обезьянам **Rhesus** и занимающихся почечными тканевыми культурами последних, вируснейтрализующие антитела были обнаружены у 18 (60%).

(2) Среди лабораторных сотрудников, получивших 2 или больше **Salk**-вакцинных дозы, антитела против вируса SV—40 выявлялись чаще, чем у непривитых лиц.

(3) Геометрическое среднее титра антител, обнаруженное у лабораторных сотрудников, превышало 23-кратно максимальный титр, найденный в контрольной группе лиц, привитых вакционой **Salk**.

(4) Автор обсуждает и предполагает возможность возникновения заражения аэрогенного происхождения в лаборатории вирусом SV—40.

HAEMAGGLUTINATION-INHIBITING ANTIBODIES TO ADENOVIRUS TYPES 8, 9 AND 10 IN PAIRED SERA OF PATIENTS WITH EPIDEMIC KERATOCONJUNCTIVITIS

Á. Jancsó

ИССЛЕДОВАНИЕ ЗАДЕРЖИВАЮЩИХ ГЕМАГГЛЮТИНАЦИЮ АНТИТЕЛ ПРОТИВ АДЕНОВИРУСОВ ТИПА 8, 9 И 10 В ПАРНЫХ СЫВОРОТКАХ БОЛЬНЫХ КЕРАТОКОНЪЮНКТИВИТОМ

А. Янчо

Изучены задерживающие гемагглютинацию антитела против аденовирусов типа 8, 9 и 10 в парных сыворотках 122 больных во время эпидемии кератоконъюнктивита, вызванного аденовирусом типа 8. Соответственно другим авторам найдено, что в сыворотках больных были задерживающие гемагглютинацию антитела не только против гомологичного, но и против 9-го и в меньшей мере против 10-го типов.

При исследовании задерживающих гемагглютинацию антител в гипериммунных сыворотках кроликов обнаружена перекрестная реакция между типами 8 и 9, но такая реакция не выявилась, с одной стороны, между типами 8 и 10, а с другой — между типами 9 и 10.

В сыворотках, взятых через 2 года после перенесения заболевания кератоконъюнктивитом, можно было еще выявить задерживающие гемагглютинацию антитела против аденовирусов типа 8, 9 и 10.

ANTIVIRAL ACTIVITY OF TWO AMINO-ETHYL-GUANIDINE AND AN EUGENOL DERIVATIVES

S. Pácsa, O. Hankovszky, K. Hideg

СВОЙСТВО ДВУХ ПРОИЗВОДНЫХ АМИНО-ЭТИЛ-ГУАНИДИНА И ОДНОГО ПРОИЗВОДНОГО ЭВГЕНОЛА ЗАДЕРЖИВАТЬ РАЗМНОЖЕНИЕ ВИРУСОВ

Ш. Пача, О. Ханковски, К. Хидег

Изучено задерживающее действие двух производных амино-этил-гуанидина и одного производного эвгенола в отношении размножения вирусов в первичных клеточных культурах почки обезьян при применении метода бляшек. Производное эвгенола умеренно, тогда как два соединения, содержащие гуанидин [Quanetidin 2-(pyridyl-ethyl) — guanidine sulfate, PYG], сильно задерживали образование бляшек у штамма полиовируса LSc 2ab, содержащего РНК-у, у штамма вируса коксаки прототипа В3 и у штамма *Herpesvirus hominis*, содержащего ДНК. Особого внимания заслуживает действие PYG-а, проявляющееся в задержке образования бляшек у вышеупомянутых вирусов уже в концентрации 10 μг/мл. Задерживающее действие PYG-а и Quanethidin-а нельзя приписывать только гуанидинному компоненту этих соединений.

UNTERSUCHUNG DER KATALASEAKTIVITÄTSÄNDERUNG VON FUCIDIN-RESISTENTEN STAPHYLOCOCCUS AUREUS-KULTUREN

E. Kovács, K. Kókai, H. H. Mazareán

ИССЛЕДОВАНИЕ ИЗМЕНЕНИЯ АКТИВНОСТИ КАТАЛАЗЫ КУЛЬТУРЫ STAPHYLOCOCCUS AUREUS, АДАПТИРОВАННОЙ К ФУЦИДИНУ

Е. Ковач, К. Кокаи, Х. Х. Мазареан

При адаптации культуры *Staphylococcus aureus* к фуцидину активность каталазы постепенно снижается или прекращается. Энзимо-индукция культуры, оказывающейся резистентной к фуцидину, не обнаруживается потому, что при адаптации равновесие процессов смещается в направлении оксидационных механизмов, не зависящих от каталазы, поэтому фуцидин не влияет на интенсивность дыхания адаптированных микроорганизмов.

BACTERIOSTATIC EFFECT OF SALIVA OF CHILDREN WITH AND WITHOUT CARIES

D. Hattyasy, I. Szabó, L. Zalai, T. Barankay

О БАКТЕРИО-ПОДАВЛЯЮЩЕМ СВОЙСТВЕ СЛЮНЫ ЛИЦ, ИМЕЮЩИХ И НЕ ИМЕЮЩИХ КАРИЕС

Д. Хаттяши, И. Сабо, Л. Залаи, Т. Баранкаи

Изучены: бактериальная флора кариесрезистентной (КР) и кариесактивной (КА) слюны, бактериальная флора кариозной полости, а также бактериостатическое действие слюны.

Бактериальная флора состоит, главным образом, из стрептококков, которые у КА-лиц особенно при аэробных, а у КР-лиц при анаэробных условиях культивируемы.

Бактериостатическое действие слюны выражено, в основном, в отношении лактобактерий и проявляется особенно сильно при наличии КР-ой слюны. Рост стрептококков задерживается КА-ой и КР-ой слюной в одинаково незначительной степени.

STUDIES ON THE ANTIGENIC STRUCTURE OF MYCOBACTERIA. I. COMPARISON OF THE ANTIGENIC STRUCTURE OF PATHOGENIC AND SAPROPHYTIC MYCOBACTERIA

S. Tuboly

ИССЛЕДОВАНИЯ, КАСАЮЩИЕСЯ АНТИГЕННОЙ СТРУКТУРЫ МИКОБАКТЕРИЙ

I. СРАВНИТЕЛЬНОЕ ИЗУЧЕНИЕ АНТИГЕННОЙ СТРУКТУРЫ ПАТОГЕННЫХ И САПРОФИТНЫХ МИКРОБАКТЕРИЙ

Ш. Тубой

Автором изучена антигенная структура штаммов: одного — человеческого, 4 — бычьего, 4 — птичьего типа палочки туберкулеза, 2 — палочки паратуберкулеза и 3 — сапрофитных микобактериальных штаммов (*M. phlei*, *M. smegmatis* и *M. minetti* по одному). Бактериальные клетки раздроблялись ультразвуковыми лучами, иммунные сыворотки получались путем иммунизации кроликов раздробленными и эмульгированными в адъюванте Фреунда антигенами. Гель-преципитационным методом **Ouchterlony** и иммуноэлектрофоретическим методом **Grabar-Williams** определено число антигенных компонентов и их электрофоретическая подвижность, а также выяснена встречаемость отдельных компонентов в других типах и видах. В человеческом типе палочки туберкулеза 10, в штаммах бычьего типа 8—10, в штаммах птичьего типа 8—9, а в сапрофитных видах микобактерий 4—6 антигенных компонента изолировались. Сравнительные исследования, проведенные с иммунными сыворотками, полученными с помощью палочек туберкулеза человеческого и птичьего типов, подтвердили, что в каждом из изученных штаммов микобактерий имеется, по крайней мере, один общий антигенный компонент. Можно определить и антигенные компоненты, которые характерны только для данного вида микобактерий. В штаммах, принадлежащих к патогенным видам микобактерий, можно выявить также антигенный компонент, который в сапрофитных микобактериях отсутствует.

ELECTRONMICROSCOPIC EXAMINATION OF KB CELL CULTURES INFECTED WITH ADENOVIRUS TYPE 12

K. Lapis

ЭЛЕКТРОННО-МИКРОСКОПИЧЕСКОЕ ИССЛЕДОВАНИЕ КЛЕТОЧНЫХ КУЛЬТУР КБ, ЗАРАЖЕННЫХ АДЕНОВИРУСОМ ТИПА 12

К. Лапиш

В ядре клеток, зараженных аденовирусом типа 12, накопляется вещество, не содержащее нуклеиновой кислоты, резистентное к пепсину, выраженно осмиофильное, электроннопоглощающее. Количество этого вещества быстро повышается, и через 36—48 часов оно образует сеть, покрывающую все ядро, а местами — гексагональные, кристаллоподобные образования.

В соседстве с этой ретикулярной сетью — вначале рассеянно, а потом по группам — появляются вирусные частицы, образуются вирусные кристаллы различной величины; бывает наоборот, когда вся ядерная субстанция заполняется вирусными частицами. По мере прогрессирования процесса разрушения клетки некоторые из вирусных частиц, формирующихся в ядре, а иногда и полные вирусные кристаллы могут появиться в цитоплазме.

Диаметр вирусных частиц, внедрившихся в Эпон клетки, был в среднем 70 мμ. Центральная часть круглых или овоидных вирусных частиц является сильно осмиофильной и электроннопоглощающей, окруженной кольцом меньшей плотности. Дезоксирибонуклеаза разлагает центральную часть, а пепсином переваривается капсид.

Автор редко наблюдал в цитоплазме зараженных клеток развитие в высокой степени организованной, богатой мембранной системы.

Изменения в клетках, вызванные аденовирусом типа 12, скорее всего похожи на таковые, описанные при заражении аденовирусом типа 5.

PHAGE-TYPING OF ESCHERICHIA COLI O124 : K72 (B17)BY THE EXAMINATION OF LYSOGENICITY

Zs. Deák

КЛАССИФИКАЦИЯ ШТАММОВ E. COLI O124:K72 (B17) ПО ТИПАМ НА ОСНОВАНИИ ИССЛЕДОВАНИЯ ЛИЗОГЕННОСТИ

Ж. Деак

С помощью выявления темперированных фагов, несённых штаммом, разработан метод определения фагового типа штаммов *E. coli* O124 : K72 (B17). Штаммы их происходят из эпидемий водного происхождения, из скопляющихся и спорадических случаев, от больных и носителей. Изолированные в различных областях Венгрии 1526 штаммов распределены на 11 типов, хорошо дифференцируемых. Фаговые типы оказались стабильными. Обнаружена взаимосвязь между отдельными фаговыми типами штаммов E. coli O124:K72 (B17) и их способностью разлагать лактозу. При изучении географического распределения штаммов установлено, что в западных и северных областях страны превалировал тип первый, а в юго-восточной части — тип третий. Метод с эпидемиологической точки зрения хорошо применяем, как это было доказано исследованиями, проведенными в течение эпидемий (в семьях и в коллективах).

INVESTIGATIONS INTO THE OLIGOSACCHARIDE DECOMPOSITION BY CANDIDA BRUMPTII (LANGERON ET GUERRA) LANGERON ET GUERRA AND PROCANDIDA GRUBYI NOVÁK ET VITÉZ

E. K. Novák, F. Kevei, B. Oláh, J. Zsolt

ИССЛЕДОВАНИЕ РАЗЛОЖЕНИЯ ОЛИГОСАХАРИДА ШТАММАМИ CANDIDA BRUMPTII (LANGERON ET GUERRA) LANGERON ET GUERRA AND PROCANDIDA GRUBYI NOVÁK ET VITÉZ

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При изучении разложения олигосахарида штаммами *Candida brumptii* и *Procandida grubyi* установлено, что оба вида дрожжевых грибков разлагают внутриклеточными энзимами сахарозу и мальтозу. Энзим обоих видов, разлагающий мальтозу, и *P. grubyi* энзим, ферментирующий сахарозу, принадлежат к типам, уже выявленным и из других дрожжей, тогда как энзим *C. brumptii*, разлагающий сахарозу, представляет собой новый, не чувствительный к ацетону тип. Выявить инвертазу не удалось ни из одного вида.

За расхождение у изученных видов спектров ассимиляции и ферментации сахара является ответственной разница, существующая между их системами, транспортирующими сахар.

OCCURRENCE OF ATYPICAL MYCOBACTERIA IN MACACUS RHESUS

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ОБНАРУЖЕНИЕ АТИПИЧНЫХ МИКОБАКТЕРИЙ У ОБЕЗЬЯН MACACUS RHESUS

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Из 66 обезьян *Macacus rhesus* и 3 обезьян **Cercopithecus** у 33 выделены 50 штаммов атипичных микобактерий. Из изолированных штаммов 46 показали дисгонический рост и размножались только при 37°, 4 росли быстро.

9 штаммов на основании фотохромогенного свойства, дисгонического роста, энзимной реакции и вирулентности для мышей определились как неизвестные до сих пор **species**. Для их обозначения авторами предложено название *Mycobacterium simiae*.

Из 16 ахромогенных штаммов изучены 6. На основании энзимной реакции 5 штаммов принадлежали к группе **avium**. Амидазный спектр 4 скотохромогенных штаммов соответствовал скотохромогенным штаммам, выделенным от человека.

Из 4-х быстро развивающихся штаммов 2 явились сапрофитными, а 2 показали высокую вирулентность для мышей.

То обстоятельство, что упомянутые микобактерии могут в мышах размножаться, делает вероятным предположение, что они в состоянии вызвать продолжительное заражение и у обезьян. Однако их патологическую роль у обезьян не могли установить.

BREAKDOWN OF AMINO ACIDS BY ENTEROBACTERIACEAE IV. GLYCINE DECOMPOSITION TEST

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РАЗЛОЖЕНИЕ ENTEROBACTERIACEAE АМИНОКИСЛОТ. IV. ТЕСТ РАЗЛОЖЕНИЯ ГЛИЦИНОМ

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Автором излагается простой, базирующийся на пробах подщелачивания и нингидрина, пригодный в практической работе метод для установления глициноразлагающей способности бактерий. При применении метода микробы, причисленные в семейство Enterobacteriaceae можно подразделить на глицино-отрицательную (Shigella, *S. paratyphi A)* и глицино-положительную *(E. coli*, Klebsiella, Citrobacter, Enterobacter, Serratia, Arizona, различные Salmonella, Proteus-Providencia) группы, однако среди членов глицино-положительных родов (species) обнаруживаются в различном количестве минус-варианты. Недельный срок наблюдения макротеста, путем постановки микротеста, может быть сокращен на сутки.

SEROLOGICAL EVIDENCE OF THE INCIDENCE OF INFLUENZA EQUINE A-1 VIRUS INFECTIONS AMONG HORSES IN HUNGARY

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СЕРОЛОГИЧЕСКОЕ ДОКАЗАТЕЛЬСТВО ВСТРЕЧАЕМОСТИ ЗАРАЖЕНИЯ ВИРУСОМ INFLUENZA EQUI А—1 ЛОШАДЕЙ В ВЕНГРИИ

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Сыворотками, взятыми в 12 группах лошадей, проводились пробы задерживания гемагглютинации с целью возможного выявления антител против вируса лошадинного гриппа А—1 и А—2. Исследования проводились и со штаммами вируса человеческого гриппа А—2 и вируса *Shope*. Из 835 обследованных лошадей 487 были гоночными. Антитела, задерживающие гемагглютинацию, были обнаружены только против подтипа *equi* А—1. В сыворотке 4-летних и более старых лошадей — независимо от группы — антитела встречались в высоком проценте. Сыворотки 2 и 3-летних жеребят исследовались в 4 группах, среди которых в двух, имевших друг с другом связи, антитела обнаруживались больше чем в одной трети сывороток, тогда как в двух других антитела практически не присутствовали. Исследования указывают на то, что virus equi А—1 4—5 лет тому назад был весьма распространенным среди лошадей, а в отдельных группах встречается и в настоящее время.

EPISOMIC ANTIBIOTIC RESISTANCE AMONG SHIGELLA STRAINS ISOLATED IN HUNGARY

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ВСТРЕЧАЕМОСТЬ РЕЗИСТЕНТНОСТИ ЭПИСОМИЧЕСКОГО ХАРАКТЕРА К АНТИБИОТИКАМ СРЕДИ ШТАММОВ SHIGELLA, ИЗОЛИРОВАННЫХ В ВЕНГРИИ

И. Кети, А. Вертени

Среди 103 штаммов Shigella, изолированных в течение 1964 и 1965 гг. и располагавших различными антибиограммами, у 102 можно было доказать эписомический характер резистентности к антибиотикам.

Проведенный с 83 штаммами «Transfer» на штамм *Serratia marcescens* в 61%, проведенная с 62 штаммами — с помощью окридин-оранжа — обработка в 93% оказались

эффективными. Результаты исследований «transfer» показал взаимосвязь с антигенной структурой штаммов.

Значительное большинство факторов R, одинаково в экспериментах «transfer» и «стирания» показало сегрегацию. Направление, частота сегрегации в экспериментах «стирания» и «transfer» или в связи со спонтанной потерей фактора R не были идентичными.

STUDIES OF THE RELATIONSHIP BETWEEN LIPID CONSTITUENTS AND BIOLOGICAL PROPERTIES OF PROTEUS BACTERIA

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ИССЛЕДОВАНИЯ В ОТНОШЕНИИ ВЗАИМОСВЯЗЕЙ МЕЖДУ ЛИПОИДНЫМ СОСТАВОМ И БИОЛОГИЧЕСКИМИ СВОЙСТВАМИ БАКТЕРИЙ ГРУППЫ PROTEUS

Л. Ваци, И. Редаи, А. Рети, Й. М. Кишш

Изучены содержание общего липоида и фосфатида, а также состав жирных кислот 18-часовой культуры 5-и различных штаммов Proteus *(P. vulgaris, P. mirabilis, P. morgani, P. rettgeri, P. inconstans.)*

Сделаны следующие выводы:

(1) В штаммах Proteus количество общего липоида колеблется между 5,5—7,5%, а количество фосфатида между — 4—4,9%.

(2) Все штаммы содержали 4 фосфатидных компонента (кардиалипин, кефалин, лецитин, лизокефалин), количественные отношения которых различаются между собой в различных штаммах.

(3) Липоиды штаммов, по существу, построены из идентичных жирных кислот. Среди жирных кислот в наибольшем количестве встречаются: миристиновая кислота, бета-гидрокси-миристиновая кислота, пальмитиновая кислота, а также жирные кислоты с циклопропановым кольцом, содержащие 17 и 19 углеродных атомов.

(4) Бросается в глаза большое количество жирных кислот с длинными углеродными цепочками, а также жирных кислот со специальной структурой и незначительное количество ненасыщенных жирных кислот.

EFFECTS OF TANNIC ACID ON DIFFERENT VIRUSES

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ВЛИЯНИЕ ТАННИНА НА РАЗЛИЧНЫЕ ВИРУСЫ

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Изучено влияние на различные вирусы водного раствора таннина, имеющегося в торговом обращении. *Herpesvirus hominis*, *Herpesvirus suis* и вирус parainfluenzae типа 3 оказались чувствительными, а полиовирусы типа 2 и 3 и аденовирусы типа 3, 4 и 6 — резистентными к действию таннина. Выглядит так, что действие таннина имеет вируцидный характер. Таннин, дозированный после заражения, не уменьшил, а наоборот, до некоторой степени повысил инфективный титр *Herpesvirus hominis*. Чувствительность клеток, предварительно обработанных таннином, снизилась в отношении герпетических вирусов. Вируцидное действие приписывается полианионной природе таннина.

THE ANTIFUNGAL COMPOUNDS OF CYNANCHUM VINCETOXICUM (L.) PERS. I. THE QUANTITATIVE ANTIFUNGAL SPECTRUM OF SUBSTANCE C-1

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АНТИФУНГАЛЬНЫЕ СОЕДИНЕНИЯ CYNANCHUM VINCETOXICUM (L) PERS. I. АНТИФУНГАЛЬНЫЙ СПЕКТР СОЕДИНЕНИЯ «С-1»

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Авторами изучено антифунгальное действие названного ими вещества «С-1», изолированного из *Cynanchum vincetoxicum* (L) *Pers*, против 40 дрожжей, 40 плесеней и 20 дерматофитонов. За исключением некоторых плесневых грибов, различные виды грибов оказались в большой степени чувствительными к действию этого вещества. Минимальная задерживающая концентрация оказалась 0,015—1 г/мл на среде бульона с глюкозой при pH 7,0.

STUDIES ON THE CONDITIONS OF INTERFERON PRODUCTION BY CELLS INFECTED WITH HERPESVIRUSES

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ИССЛЕДОВАНИЯ В СВЯЗИ С ИНТЕРФЕРОН-ОБРАЗУЮЩЕЙ СПОСОБНОСТЬЮ ГЕРПЕТИЧЕСКИХ ВИРУСОВ

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Авторами изучено во вторичных человеческих фибробластных клеточных культурах образование вирусподавляющих веществ, возникающих при размножении *Herpesvirus hominis*, *Herpesvirus varicellae* и *Cytomegalovirus*. Образование задерживающего размножение вируса вещества обнаруживалось при размножении всех трех видов вирусов. Задерживающее вещество обнаруживалось в наибольшем количестве в течение размножения *Herpesvirus varicellae* в меньшем — при размножении Cytomegalovirus и в наименьшем количестве — при размножении *Herpesvirus hominis*. Для титрования задерживающего вещества применялись вторичные культуры человеческих фибробластов, Hela и непрерывная культура почки обезьян III/1. При применении последней культуры обнаруживалось наиболее выраженное задерживающее действие. Задерживающий фактор оказался интерфероном или же веществом, подобным интерферону. Была найдена обратная взаимосвязь между количеством интерферона, образованного 3 вирусами, и количеством вируса, освобожденного из клеток в процессе размножения.

ANIMAL EXPERIMENTS CONCERNING THE AETIOLOGY OF VIRAL HEPATITIS

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ОПЫТЫ НА ЖИВОТНЫХ В СВЯЗИ С ЭТИОЛОГИЕЙ ВИРУСНОГО ГЕПАТИТА

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Авторами сделаны попытки репродуцировать исследования Бертока и сотрудников, то есть сенсибилизировать крысы к заражению вирусом человеческого гепатита путем дачи метионин-дефицитной диэты, содержащей 0,3% (ежедневная доза 23—25 мг) этионина. Содержавшиеся таким образом животные (первая группа), а также животные, содержавшиеся на нормальной диэте (третья группа), прививались материалами, полученными от больных, страдавших острым гепатитом. Вышеупомянутые, а также непривитые адекватные контрольные животные (вторая и четвертае группы) содержались

под наблюдением. Пробы их сывороток исследовались с целью определения наличия гемагглютинирующего фактора против человеческих эритроцитов, обработанных танином, и анализировались также содержание белка и состав.

Относительный уровень гаммаглобулинов в сыворотке животных первой группы и гемагглютинирующая активность значительно отличались от соответствующих данных остальных животных. С другой стороны, обнаруживались желтуха, а также падеж и среди непривитых и получавших этиониновую диэту животных (II группа), и удалось выявить гемагглютинирующий агент и из печени привитых, но содержавшихся на нормальной диэте животных (III группа). Таким образом, авторы не могли ни подтвердить, ни опровергнуть результаты Бертока и сотрудников.

HISTOPATHOLOGICAL FINDINGS IN ANIMAL EXPERIMENTS ON THE AETIOLOGY OF VIRAL HEPATITIS

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ГИСТОПАТОЛОГИЧЕСКИЕ ИССЛЕДОВАНИЯ В ЭКСПЕРИМЕНТАХ НА ЖИВОТНЫХ ОТНОСИТЕЛЬНО ЭТИОЛОГИИ ВИРУСНОГО ГЕПАТИТА

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Исследовательская группа Кафедры Гигиены Будапештского Медицинского Университета повторила исследования Бертока и сотрудников на крысах, относящиеся к переносу вируса эпидемического гепатита; из фигурирующих в опыте животных патогистологически исследовались печени 34 крыс. В печени животных, содержавшихся на нормальной синтетической диэте и заражённых только «вирусом гепатита» патологические изменения не были обнаружены. Изменения, обнаруженные в печени крыс, кот рые содержались на этиониновой диэте и не заражались, и изменения в печени животных, которые при этиониновой диэте заражались, не отличались друг от друга. При сравнении изменений в печени, обнаруженных в связи с эпидемическим гепатитом человека и собак, с изменениями в печени крыс, найденными после дачи этионина и заражения «вирусом гепатита», существенной разницы не было. У животных, содержавшихся на этиониновой диэте, без зависимости от того, заражались они или нет, в печени не было обнаружено разницы, указывающей на какие-нибудь, будто бы вызванные «вирусом гепатита», специфические патогистологические изменения.

AGAR-DIFFUSION METHOD FOR THE SCREENING OF ANTICANCER SUBSTANCES BY PHAGE INDUCTION

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АГАР-ДИФФУЗИОННЫЙ МЕТОД ДЛЯ «SCREENING» ВЕЩЕСТВ, ИМЕЮЩИХ ПРОТИВООПУХОЛЕВОЕ ДЕЙСТВИЕ, С ПОМОЩЬЮ ФАГОВОЙ ИНДУКЦИИ

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Авторами разработан полуколичественный агар-диффузионный метод для «*screening*» антикарциногенных веществ с помощью фаговой индукции. Метод прост и пригоден и для массовых исследований, основывается на следующих принципах: смесью, изготовленной из соответствующего соотношения лизогенного и индикаторного штаммов, заражается плотная питательная среда, и в окружности отверстий, всверленных в агар, на действие активных веществ значительно повышается количество бляшек.

SHIGELLA FLEXNERI SEROVARIANTS CONTAINING COMBINATIONS OF TYPE ANTIGENS

L. Kerekes

СЕРОВАРИАНТЫ SHIGELLA FLEXNERI, СОДЕРЖАЩИЕ НЕКОТОРЫЕ ТИПОВЫЕ АНТИГЕНЫ

Л. Керекеш

На основании изолирования почти 100 штаммов *Shigella flexneri*, содержащих некоторые типовые антигены, автор знакомит с серологическими, биохимическими и эпидемиологическими свойствами вариантов. Антигенная структура их: S, 1, (III), (IV): 6, (3, 4) или (S), (III), (IV): 6, (3, 4). Они содержат и термолабильный антиген E. Большинство из них ферментирует маннит и не образует индол. Один штамм сбраживает и рамнозу. Редко обнаруживаемые, разлагающие сорбит варианты в каждом случае образовывали индол. Серологические и биохимические вариации не зависят друг от друга. Морфология их колоний напоминает *Shigella flexneri* 1b. У морской свинки вызывают keratoconjunctivitis shigellosa.

Кроме спорадических заболеваний, варианты вызывали и незначительные эпидемии. На территории их географической встречаемости частота заражений *Shigella flexneri* 1b была вдвое выше, чем в других местах. В области Солнок, где автором проводилис наблюдения, приблизительно 2,7% изолированных типов *Sh. flexneri* составили эти штаммы, значит, они обнаруживаются в таком же проценте, как штаммы более редко наблюдаемых серотипов.

IMMUNOLOGICAL STUDIES ON SHIGELLOSIS BY THE MOUSE MODEL TECHNIQUE. I. ANTIINFECTIVE IMMUNITY OF ACTIVELY IMMUNIZED MICE

K. Rauss, I. Kétyi

ИММУНОЛОГИЧЕСКИЕ ИССЛЕДОВАНИЯ, ПРОВЕДЕННЫЕ НА ЭКСПЕРИМЕНТАЛЬНОЙ МОДЕЛИ «МЫШЬ-SHIGELLOSIS» I. АНТИИНФЕКТИВНЫЙ ИММУНИТЕТ АКТИВНО ИММУНИЗИРОВАННЫХ МЫШЕЙ

К. Раушш, И. Кети

Некоторые бактерии вирулентных мутантов штаммов *Sh. flexneri* типа 2a и 3 вызывают продолжительное, бессимптомное выделение у мышей, не имеющих флору *E. coli*. С помощью перорально или парэнтерально проведенной активной иммунизации представляется возможным препятствовать развитию бессимптомного выделения. Иммунитет является типо-специфичным и зависит от качества антигена, а не от фактора (факторов) вирулентности, содержавшихся в вакцине.

Иммуногенный эффект авирулентных живых бактерий при пероральном применении не превышает антигенность убитых шигелл. Перорально наиболее эффективным оказался коллоидальный антиген.

Парэнтеральная иммунизация является более эффективной, чем пероральный метод. Наиболее эффективным оказался, и при применении нашей модели, адсорбированный прививочный материал. Иммунитет можно поддерживать перорально введёнными небольшими дозами антигена.

Изученный на экспериментальной модели и проявляющийся в задержке выделения иммунитет, повидимому, можно оценивать при изучении природного и искусственного иммунитета против дизентерии, и он находится в соответствии с иммунологическими закономерностями, познанными с помощью других методов.

IMMUNOLOGICAL STUDIES ON SHIGELLOSIS BY THE MOUSE MODEL TECHNIQUE. II. ANTIINFECTIVE IMMUNITY OF PASSIVELY IMMUNIZED MICE

K. Rauss, I. Kétyi

ИММУНОЛОГИЧЕСКИЕ ИССЛЕДОВАНИЯ, ПРОВЕДЕННЫЕ НА ЭКСПЕРИМЕНТАЛЬНОЙ МОДЕЛИ «МЫШЬ-SHIGELLOSIS» II. АНТИИНФЕКТИВНЫЙ ИММУНИТЕТ ПАССИВНО ИММУНИЗИРОВАННЫХ МЫШЕЙ

К. Раушш, И. Кети

Пассивно (сывороточными или копро-антителами), путем подкожной и пероральной иммунизации одинаково можно препятствовать продолжительному, бессимптомному выделению у мышей. На основании этих исследований можно установить, что гуморальный фактор иммунитета имеет решающее значение в иммуномеханизме «мышь-Shigellosis».

Защищающий эффект сывороточных и копро-антител является равноценным. Между этими двумя типами антител качественной разницы нет, различать их можно только на основании локализации.

Перорально применяемая пассивная иммунизация является наиболее эффективной; значение местной концентрации антител вытекает из патомеханизма Shigellosis.

PROPERTIES OF PENICILLIN ACYLASE ISOLATED FROM *E. COLI*

A. Szentirmai

СВОЙСТВА ПЕНИЦИЛЛИНАЦИЛАЗЫ, ИЗОЛИРОВАННОЙ ИЗ E. COLI

А. Сентирмаи

Автор экстрагировал из E. coli ацилазу, разлагающую и синтезирующую Г-пенициллин, и с помощью обычных методов (фракционирование сернокислым аммонием, кальциумфосфат-гелевая адсорбция, хроматография через целлюлозную колонку DEAE) очищал сорокократно. Очищенным энзимом определены кинетические данные разложения и синтеза Г-пенициллина, далее изучена специфичность субстрата. Выявлено, что очищенный энзим стереоспецифически и с большой активностью разлагает производные N-фенил-ацетил-кислоты.

Упомянутые экспериментальные данные указывают на то, что энзим играет роль процессах трансацилирования обмена веществ. Результаты исследований в отношении вадержки объясняют одновременно влияние производных фенил- и феноксиуксусной зислоты на образование энзима: эти кислоты, задерживая деятьность энзима, вызывают кепрессию.

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