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MOTION PICTURE STUDY OF THE RESPONSE OF CULTURED PERITONEAL MACROPHAGES TO THE INVASION OF ENDOZOITS OF TOXOPLASMA GONDII, RH STRAIN

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(Received June 20, 1978)

A motion picture study of macrophage culture infected with the endozoits of *Toxoplasma gondii* revealed an enhanced locomotor activity in affected cells: regular contractions of the cell resulting in an incomplete extrusion of the parasitophorous vacuole or host-cell destruction, formation of excessive undulating membranes and pinocytotic vesicles.

The morphological aspects of the interactions of toxoplasmas and mononuclear phagocytes have been studied mainly by means of cytochemical and electron microscopic investigations of infected cultures of peritoneal macrophages [1–6]. Vital studies of infected macrophages suggest that the cell layer is quickly damaged by the toxic products of the toxoplasmas contained in the peritoneal exudate [5]. Application of medium doses allows to observe the cells during several days [4, 5]. There are no data in the literature on the locomotor reactions of living mononuclear phagocytes involved in challenge. The aim of the present study was to follow the response of living mononuclear phagocytes to the entry and multiplication of toxoplasma endozoits in them. Time-lapse motion pictures were employed to study the locomotor activity of cells.

Materials and methods

A suspension of endozoits of *Toxoplasma gondii*, RH strain, was used for challenging a 48 hr culture of mouse peritoneal macrophages in a Karrel flask. The challenging dose was one parasite per macrophage [5]. An inverted microscope MBI-12 (LOMO, Leningrad, USSR) with a $90\times/1.25$ objective was used for observation and filming, at 37°C constant temperature. Intervals between shots ranged from 1 to 5 s.

Results

At the dosage used, all toxoplasmas were seen to penetrate into macrophages in 6–8 hr. At 12–15 hr, many cells contained colonies consisting of 2–8 toxoplasmas; larger colonies were less frequent. After 20–24 hr, some cells filled with toxoplasmas began to degrade. Some parasites entered neighbouring

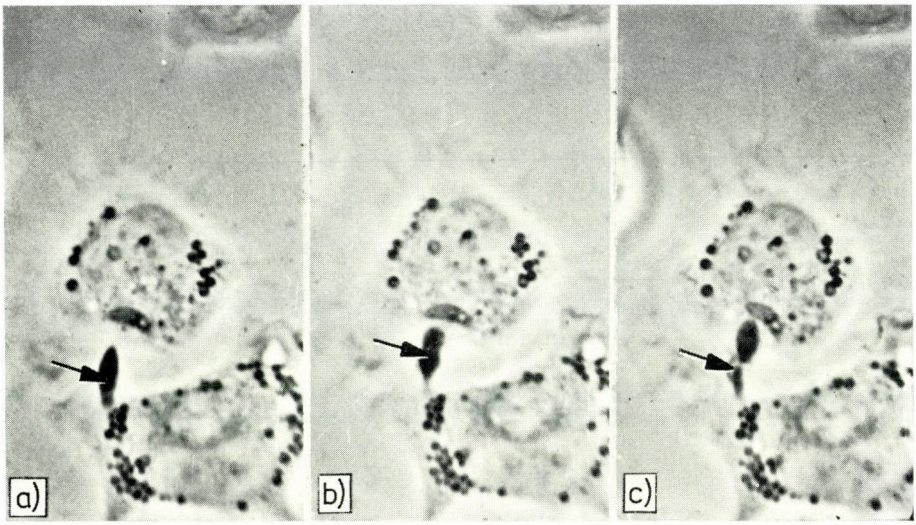

Fig. 1. Successive stages of toxoplasma (arrow) penetrating into a macrophage: (a) attachment; (b, c) entry, 24 hr postchallenge. Intervals between pictures 5 s

non-infected macrophages or those containing parasitic colonies. By 48 hr, the infected cells began to die on a large scale and by day 3 the cell layer appeared to be destroyed.

Locomotor activity was apparent both in the macrophages or intact cultures and in intact cells of infected cultures. It took the form of combined amoeboid and gliding movements. It involved the formation of microspikes by which the cells became attached to the glass, and undulating membranes. The latter formed the leading edge of moving cells. Pinocytotic vesicles formed near the undulating membranes. In intact macrophages, undulating membranes had formed along the cell periphery; they never occurred on the top cell surface.

Penetration of toxoplasmas into macrophages and colony formation was followed by a sharp intensification of all forms of locomotor activity of the cells. During the entry of viable (i.e. capable of independent movement) toxoplasmas, the stages of attachment and penetration proper could clearly be distinguished. The duration of the attachment to the cell of the anterior pole of the parasite, varied within a wide range. It appeared to be determined by the functional state of the cell rather than by the activity of the parasite itself. It was the shortest, 1 to 5 s, when toxoplasmas became attached to mobile cells with a highly pronounced undulating membrane (Fig. 1). In contracted spherical cells which became smooth without undulating membranes, the attachment process took sometimes as long as 20–30 min and even more (Fig. 2). One toxoplasma remained attached to the surface of a contracted macrophage for over an hour, continuing to spin. Subsequently, it was detached from

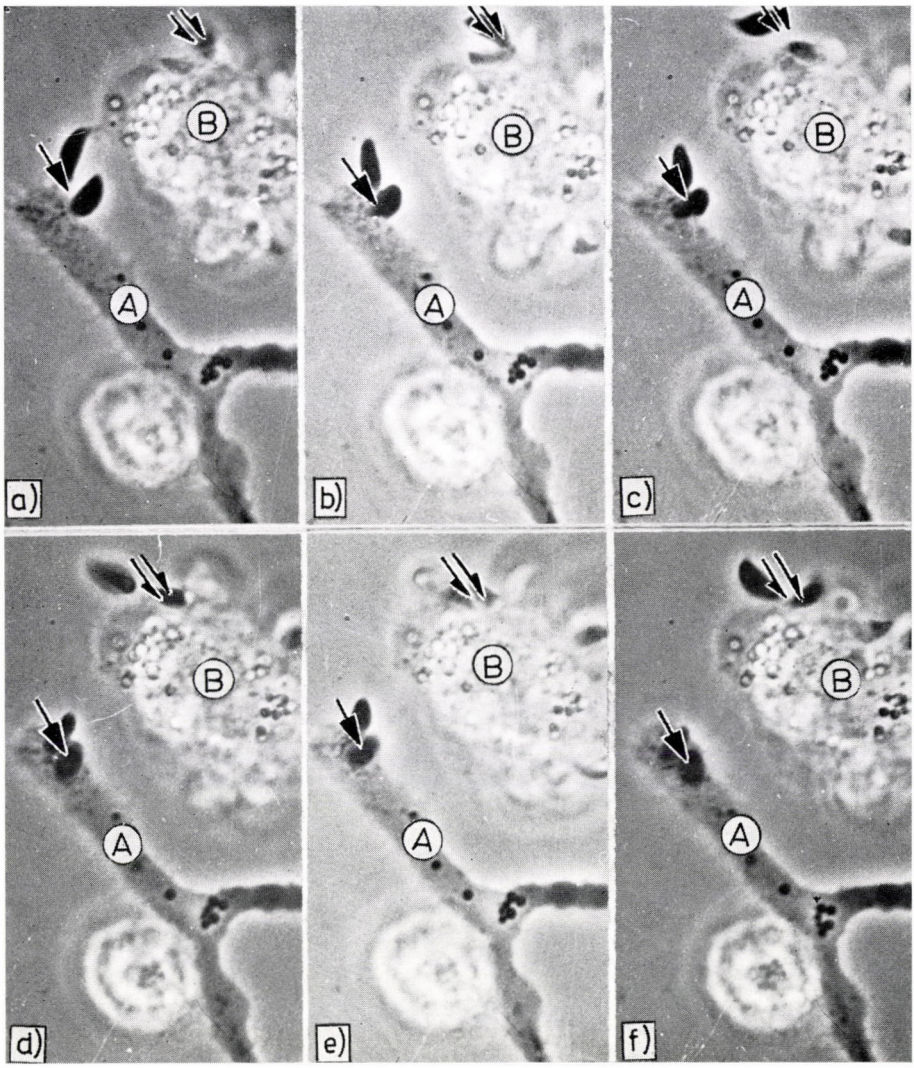

Fig. 2. Attachment and entry of toxoplasmas into macrophage (A) and attachment to a contracted one (B). Successive stages of penetration of two parasites (arrow) into cell "A". The toxoplasmas which had attached to cell "B" (double arrow) continued to spin but could not enter into the cell; 24 hr postchallenge

this cell and quickly entered a neighbouring cell, which had a vigorously undulating membrane.

Penetration of toxoplasmas was complete in 5–15 s. It involved local protuberances of the macrophage ectoplasm, which enveloped the parasite, thus forming a phagocytic vacuole. As the parasite penetrated further into the cell, it became closely enveloped by the membranes of the host cell (Figs 1b, 2c and e).

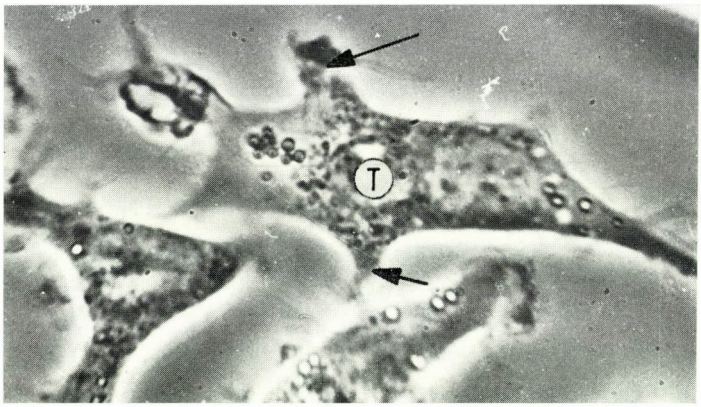

Fig. 3. Infected macrophage with additional undulating membranes (arrows) near parasitophorous vacuole, containing toxoplasmas (T); 24 hr postchallenge

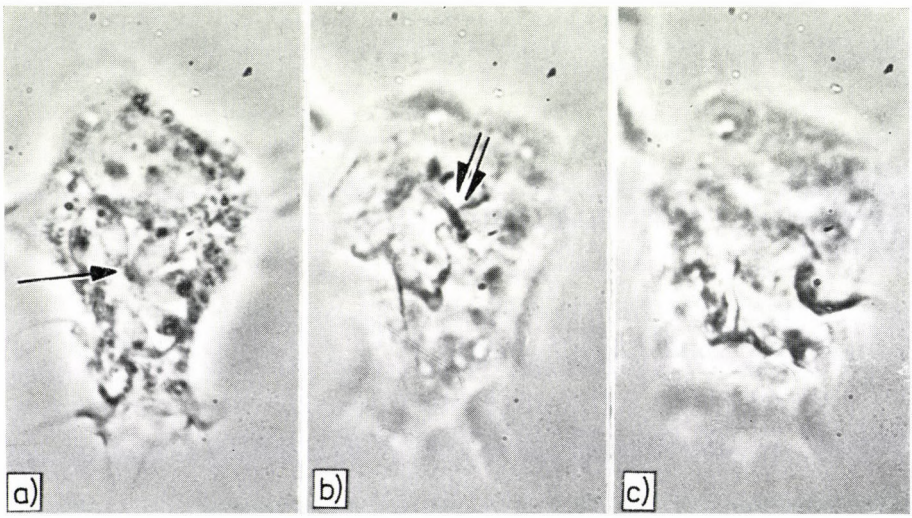

Fig. 4. Infected macrophages, 24 hr postchallenge. Different microscope focussing: (a) parasitophorous vacuole containing toxoplasmic colony (arrow); (b, c) surface of macrophage with undulating membranes and large pinocytotic vacuole (double arrow); 24 hr postchallenge

The infected cells displayed the following forms of locomotor activity which did not occur in intact cells: formation of undulating membranes in the region of the phagocytic vacuole and regular contractions of the macrophage resulting in the extrusion of the phagocytic vacuole. More undulating membranes formed along the periphery (Fig. 3) and on the surface of macrophages (Fig. 4) so that the whole surface continued to undulate. Numerous pinocytotic vesicles were also formed; some of them were large and easily distinguished under the phase-contrast microscope (Fig. 4b).

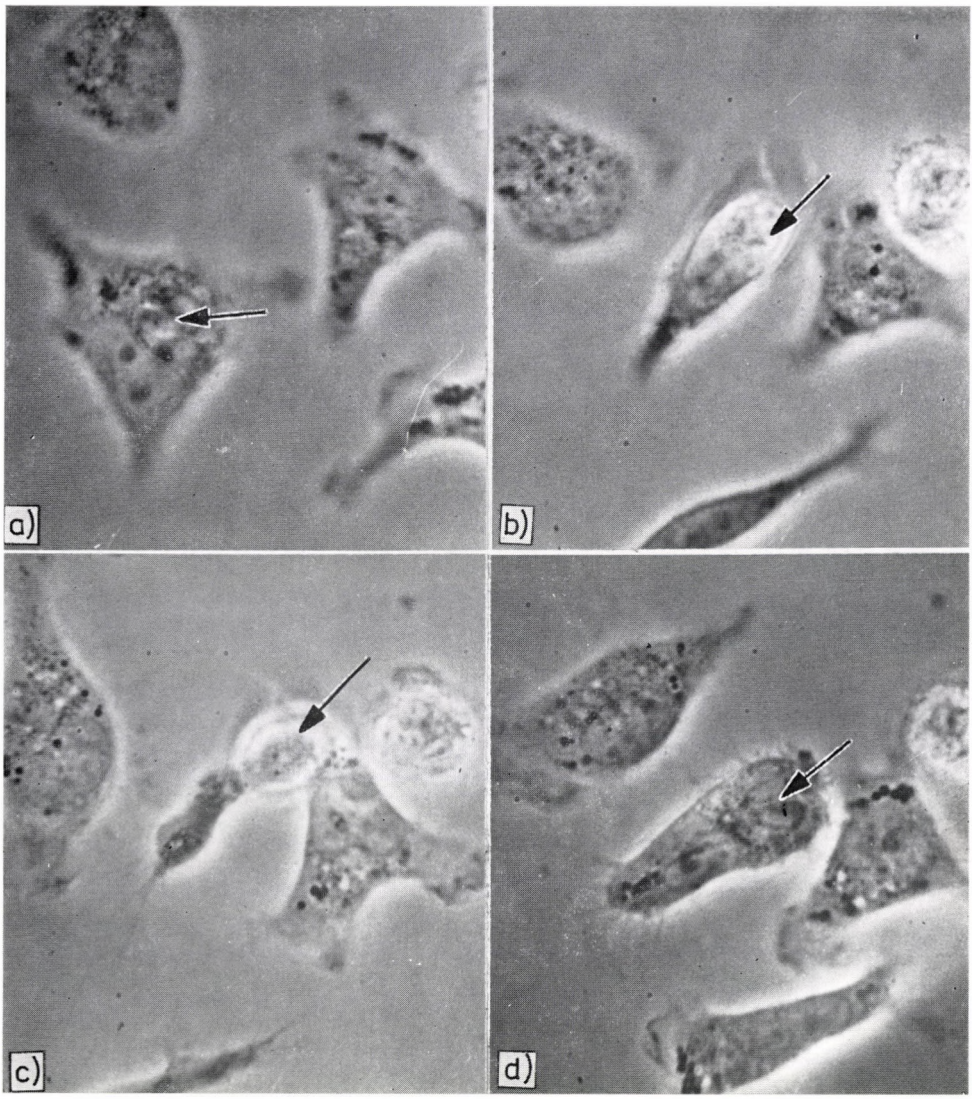

Fig. 5. Successive stages of contraction of infected macrophage, 24 hr postchallenge: (a) parasitophorous vacuole (arrow) is seen distinctly when the cell is expanded; (b, c) extrusion of parasitophorous vacuole (arrow) during cell contraction; (d) stage of relaxation

When infected cells contracted, they became spherical and detached themselves partly from the glass. Contractions lasted from several minutes to 2–3 hr, and the colony-containing vacuole was extruded from the cell (Fig. 5), maintaining, however, its integrity and contact with the cell due to the formation of a narrow cytoplasmic bridge. On relaxation of the cell, the colony-containing vacuole returned into the cell.

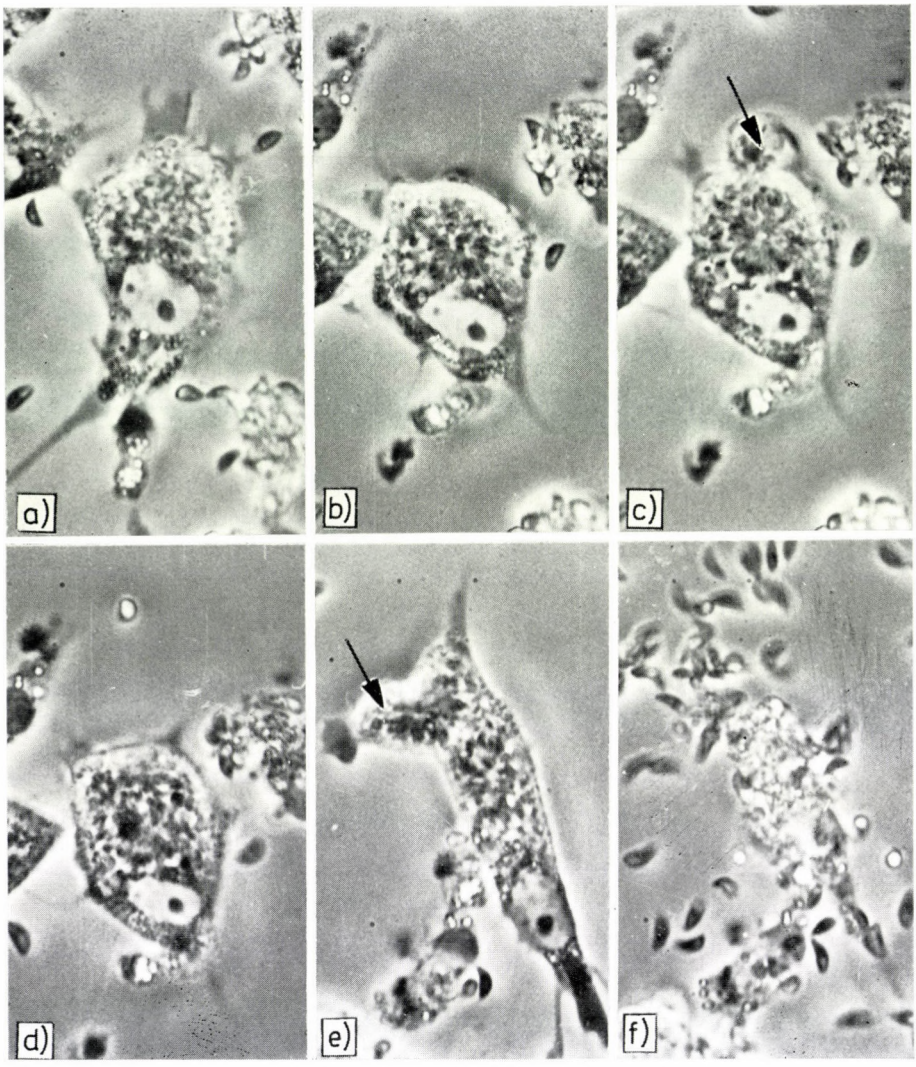

Fig. 6. Two successive contractions of heavily infected macrophage, 48 hr postchallenge. Hernia-like bulges (arrows) of parasitophorous vacuole forming during each contraction (c and e). Cell destruction after second contraction (f)

During the first 24 hr postchallenge, it was possible to observe 4–5 contraction waves of the cell when the parasitophorous vacuole was extruded. Whenever the vacuole was large, it broke during a contraction, which caused death of the host cell.

The locomotor activity of infected macrophages was noticeably reduced 48 hr after challenge. Cell movements became slow, contractions occurred regularly and involved the formation of small herniated bulges on the para-

sitophorous vacuoles (Fig. 6). As a rule, cells filled with parasites disintegrated during one of the contractions. After 48 hr, nearly all the macrophages were destroyed.

Discussion

The penetration into macrophages of toxoplasma endozoits is a complicated process. It is determined by the activity of both the parasite and the host-cell and, particularly, by the ability of the latter to form undulating membranes and bulges which envelop the intruder [7, 8]. Our vital investigations are in agreement with the electron microscopic findings [9] which showed that before entering the host-cell, viable toxoplasmas had been attached to it only by their anterior pole. Attachement by the posterior pole or by a lateral surface [10] occurs only with damaged parasites deprived of independent motility.

An enhanced locomotor activity of affected cells should be considered a sign of the activation of mononuclear phagocytes, typical of toxoplasmic invasion [11].

The biological significance of various forms of locomotion may be different. The regular contractions of the cell, extruding the parasitophorous vacuole, seem most likely to be a response intended to eliminate the intruder.

The response, however, is useless as the parasitophorous vacuole is either preserved inside the cell or destroyed. In the latter case, the host cell, too, is destroyed. Whatever its cause, the reaction needs much energy. The other type of locomotion, the vigorous movements of infected cells with formation of excessive undulating membranes, may be regarded as a form of adaptation to compensate for the spent energy. This view is supported by the formation of numerous pinocytotic vesicles in the undulating membranes, which points to an intensive uptake of metabolites from the nutrient medium. The high locomotory and pinocytotic activities of the infected cells are observed only at low challenge dosage (ratio of 1 parasite per 1 macrophage). Challenge with high doses damages the macrophages [12], and inhibits their pinocytotic activity [13].

In our experiments even at an optimum parasite-cell challenge ratio the subsequent multiplication of toxoplasmas depletes the energy reserves of macrophages, as indicated by the slowing down of their locomotor activity by the end of the experiment. Our data, however, show that the decay of macrophages filled with toxoplasmas is due not only to the activity of the parasites but also to the contractions of the host cell, which make the vacuole to rupture and thus cause death of the cell.

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Acta microbiol. Acad. Sci. hung. 27, 9—21, 1980

VITAL FLUORESCENCE MICROSCOPY OF LYSOSOMES IN CULTURED MOUSE PERITONEAL MACROPHAGES DURING THEIR INTERACTIONS WITH MICROORGANISMS AND ACTIVE SUBSTANCES

III. INTERACTIONS OF MACROPHAGES WITH ENDOZOITS OF TOXOPLASMA GONDII RH STRAIN AND THEIR SOLUBLE SUBSTANCE

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(Received June 20, 1978)

Macrophages with lysosomes pinpointed by quinacrine-induced fluorescence were infected with the endozoits of *Toxoplasma gondii* RH strain (peritoneal exudate of infected mouse), or treated with liquid (acellular) fraction of the same exudate. Dead toxoplasmas ingested by macrophages come into contact with the stained lysosomes of the cell and acquire a diffuse fluorescence. Viable toxoplasmas do not give fluorescence, which means that they do not come into contact with lysosomes, either primary or secondary. This supports the hypothesis that toxoplasmas can prevent lysosomes from fusing with the phagosomes of the host cell. Moderate doses of soluble products of toxoplasmas contained in peritoneal exudate cause an excessive output of macrophage lysosomes which points to the activition of macrophages; high doses of challenge inhibit the phagocytosis of toxoplasmas and damage macrophages. The pathogenicity of toxoplasmas due to their ability to inhibit the fusion of lysosomes and phagosomes and the cellular action of their soluble products is discussed.

In acute toxoplasmic invasion, both natural and experimental, the endozoits of toxoplasmas are known to parasitize in mononuclear phagocytes: peritoneal macrophages, Kupffer's cells of the liver, reticular cells of lymphoid tissue, and histiocytes [1, 2]. When toxoplasmas multiply, they destroy these cells causing necrosis of the surrounding tissue and general intoxication. This effect is attributed to the action of the toxic products of the parasite [1, 3, 4]. Therefore, elucidation of the pathogenicity of the endozoits of toxoplasmas calls for study of their interactions with mononuclear phagocytes. Particular attention should be given to investigations in the mechanism by which parasites withstand the defences of these cells as well as the establishment of what should be considered the signs of the action which the toxic products of toxoplasmas are supposed to exert on the affected cells.

The present study is concerned with the responses of the lysosomal apparatus of cells to the action of toxoplasmas and their toxic products, because lysosomes constitute the main effector apparatus on mononuclear phagocytes [5].

The basic data on the interactions between toxoplasmas and the lysosomes of mononuclear phagocytes were obtained in earlier experiments with cultured

peritoneal macrophages with thorotrast-labelled lysosomes [6]. Electron microscopy revealed that thorotrast-labelled lysosomes do not fuse with phagosomes containing viable toxoplasmas. It was therefore concluded that living toxoplasmas prevent lysosomes from fusing with phagosomes, thus avoiding contact with lysosomal enzymes.

Such a technique reveals, however, the interaction of the parasite with secondary lysosomes only, because it is precisely in these lysosomes that thorotrast is accumulated [7].

There is no evidence in the literature concerning the immediate action of the toxic products of toxoplasmas on cells.

Cultured peritoneal macrophages were used in this study with cells of established epithelial HeLa cell-line for comparison. Lysosomes were identified by vital fluorescent aminoacridine–quinacrine staining [8]. A preliminary investigation [9] showed that the combined application of fluorescence and phase contrast microscopy allows to identify both lysosomes and toxoplasmas and to assess the condition of lysosomes in the whole population of macrophages. It is also known [10] that aminoacridines may be effectively used for identification of lysosomes in living toxoplasmas.

Materials and methods

Cell culture. The peritoneal macrophages of non-elicited mice were obtained and cultured as described earlier [11].

Toxoplasmas and their soluble products. The abdominal cavity of mice sacrificed 3 days after intraperitoneal challenge with the endozoits of strain RH toxoplasmas was washed with medium 199. Toxoplasmas in the suspension were counted in a glass chamber. To identify lysosomes in the toxoplasmas, a sample of the suspension was incubated in quinacrine-containing medium for 1 hr. A drop of the suspension was placed under a coverslip and examined in the fluorescence microscope. Soluble products of toxoplasmas were obtained by centrifuging the suspension at 5000 g for 15 min, and the supernatant was injected intravenously to mice. Macrophages were incubated in the presence of soluble products of toxoplasmas for 2–72 hr.

Fluorescence pinpointing of lysosomes, and staining of preparations, reactions to acid phosphatase, and fluorescence microscopy were performed as described earlier [11]. Examinations were carried out within 30 min to 48–72 hr after challenge. Preparations to be examined within the first 6 hr were treated with quinacrine 1 hr before challenge. Preparations to be examined after 24 and 48 hr were stained with fluorochrome either 1 hr before challange or 1 hr before examination, and the results were compared.

For a quantitative assessment of the condition of the lysosomes of macrophages in each population, the numbers of cells with a high, medium and low level of red granules and cells without red granules were counted [11].

Control. A 24 hr culture of HeLa cells was infected with toxoplasmas. The techniques of challenge and examination were identical as those used in the experiments with infected macrophages [12].

Results

Quinacrine staining of toxoplasma suspension. It appeared that the same concentration of quinacrine (2 μg/ml) is sufficient for identification of lysosomes in toxoplasmas and macrophages [11]. The fluorescence of lysosomes (1–2

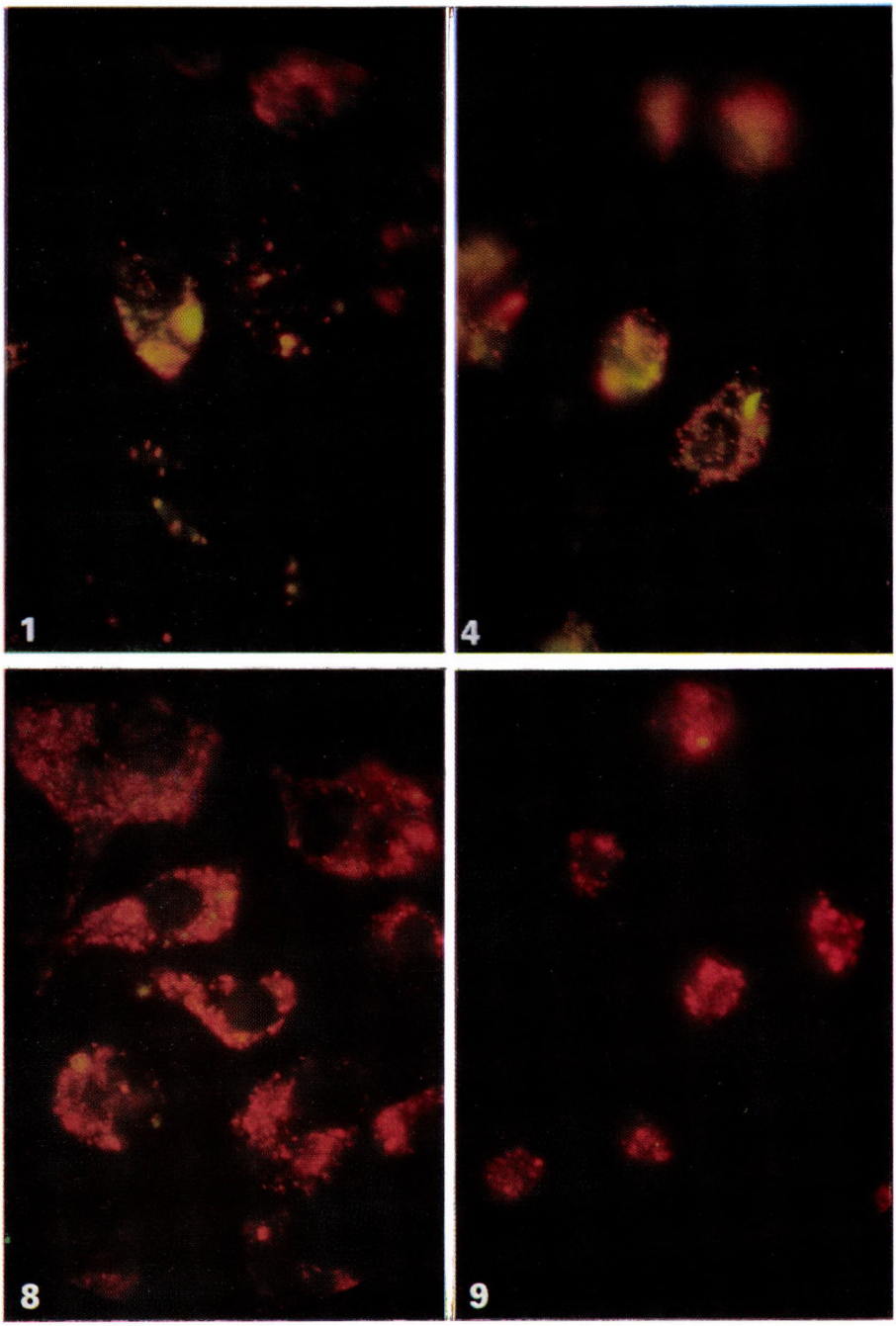

Fig. 1. Free toxoplasmas (centre) and a macrophage in peritoneal exudate of infected mouse treated by quinacrine. Elective red fluorescence of lysosomes of macrophage and parasites ×120

Fig. 4. Green and orange fluorescence of dead toxoplasmas in macrophages with quinacrine-pinpointed lysosomes. Three hr after challenge ×120

Fig. 8. Macrophage culture after a 48 hr incubation with soluble products of toxoplasmas, 1 hr after staining with quinacrine. Excessive output of lysosomes ×120

Fig. 9. Control culture of macrophages with normal lysosomes content in cells ×120

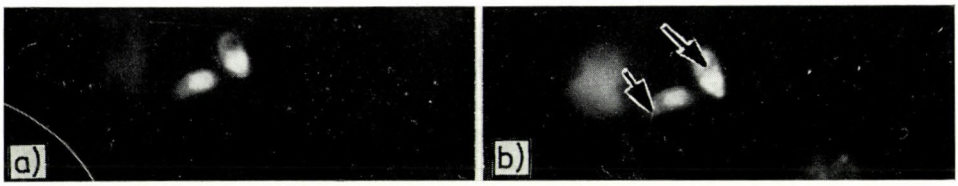

Fig. 2. Toxoplasmas isolated from quinacrine-stained suspension. Fluorescent picture taken through filters selectively transmitting either green (a) and red and green (b) light. Lysosomes (arrows) fluoresce red ×1200

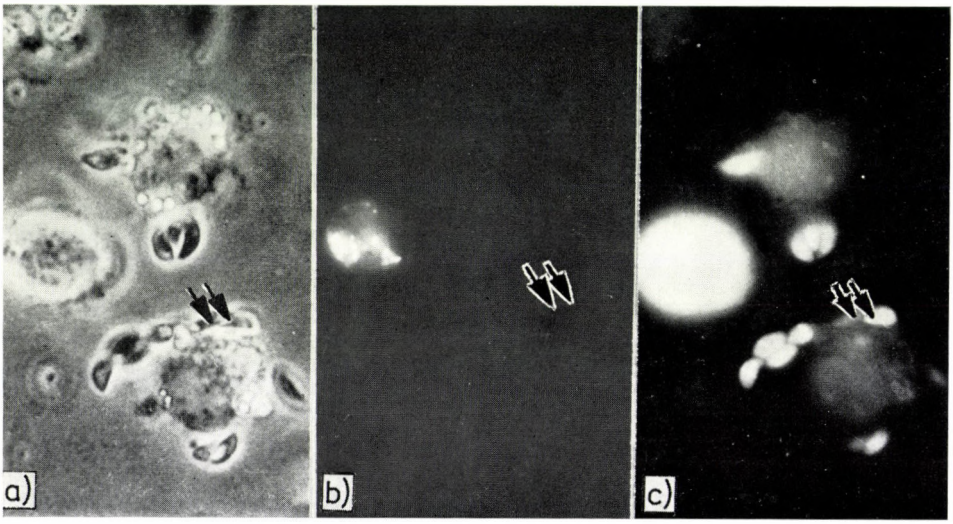

Fig. 3. Macrophage culture 30 min after infection with a high dose of toxoplasmas. Phase contrast (a) and fluorescent pictures taken through filters selectively transmitting either red (b) or green (c) light. Some macrophages degraded; red fluorescence of lysosomes is seen only in one macrophage (b) and two toxoplasmas (arrows). Bright green fluorescence of cytoplasm of macrophages and toxoplasmas (c). Toxoplasmas are attached to cell surfaces but do not penetrate through them ×800

red inclusions at one of the poles, Figs 1, 2) was observed in 40–60% of the toxoplasmas of peritoneal exudate. At a higher concentration of quinacrine (5 μg/ml), the cytoplasm of parasites showed red fluorescence and their nuclei fluoresced green. Strugger's effect (diffuse red fluorescence of the parasite, its nucleus included), considered a sign of cell death [13] was not observed until the concentration was raised to 10 μg/ml. It was recorded in 20–30% of the toxoplasmas contained in the peritoneal exudate.

Infection of macrophages. When challenging, the following ratios were used: 1 parasite per macrophage (low dose); 10 parasites per macrophage (medium dose); and 100 parasites per macrophage (high dose). Challenge with high and medium doses caused the loss of lysosomes both in infected and in

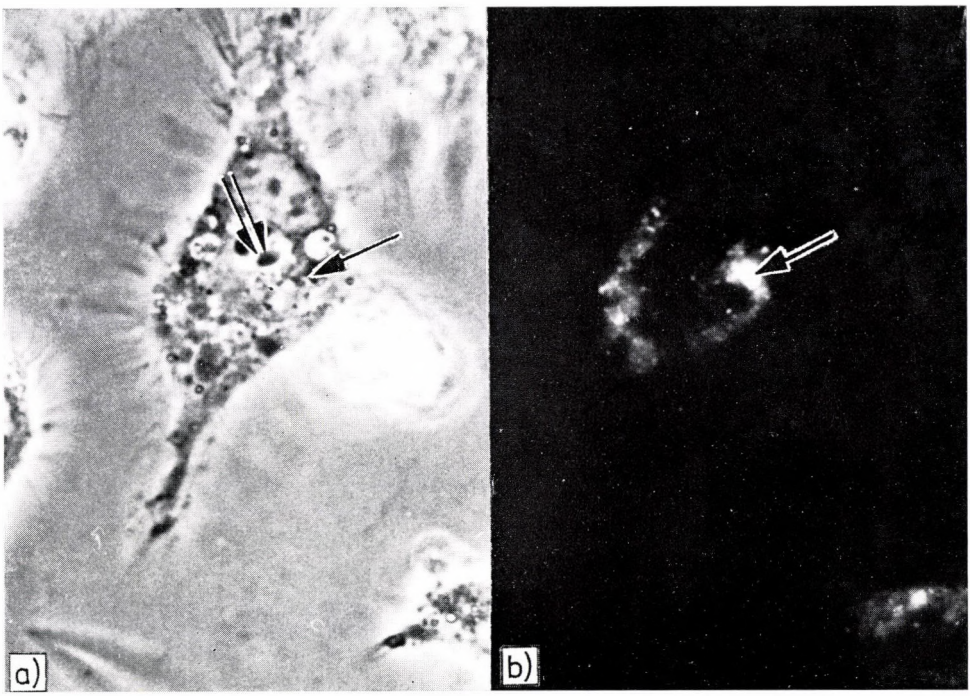

Fig. 5. Fluorescent (a) and phase contrast (b) photomicrographs taken 3 hr postchallenge and 4 hr after staining with quinacrine. Fluorescence of lysosomes of a macrophage and an ingested killed toxoplasma (arrow). Dividing parasites in the vacuole of macrophage (double arrow) do not show fluorescence × 600

intact macrophages (Table I). Medium doses caused a considerable destruction of the cell layer after 24 hr. Viable cells contained large colonies of the parasites. When high doses were employed, the toxoplasmas attached to the macrophage surface and hardly penetrated into them (Fig. 3). Cell layer was destroyed in a few hours. At low dosage (1 : 1), toxoplasmas were found to penetrate rapidly into the cells. At 4–6 hr some toxoplasmas multiplied inside the cells, and numerous parasitic colonies could be seen inside macrophages within 24 hr. Some cells filled with toxoplasmas became disintegrated. The greater portion of the cell layer appeared to be destroyed within 48 hr.

When the fluorescence-phase contrast technique was used for studying living cultures, extracellular toxoplasmas could be identified by means of phase contrast only. Some toxoplasmas, which were inside macrophages, showed fluorescence (Fig. 4). This points to their contact with the labelled lysosomes of macrophages. These parasites fluoresced green at first and then their colour gradually changed to red. Fluorescing parasites were observed mainly within the first hours after challenge; they did not seem to divide and disappeared completely after 24 hr.

During the first hour postchallenge some intracellular toxoplasmas did not fluoresce and showed division in parasitophorous vacuoles (Fig. 5). After 24 hr nonfluorescing parasitic colonies could be observed in nearly 40% of the macrophage population.

Table I

Changes in content of cells with different red granule content in population of macrophages after toxoplasma infection

Duration of cell cultivation after infection, hr	Macrophage cultures	Content of cells with different fluorescence intensity (conventional units) in macrophage cultures (per cent)*				
		High intensity	Medium intensity	Low intensity	Without granules	Mean intensity 100 cells
2	Without infection	46	41	13	0	593
	Infected 10 parasites per 1 macrophage	8	24	62	6	214
	Infected 1 parasite per 1 macrophage	19	45	36	0	361
24	Without infection**	33 / 29	47 / 50	20 / 21	0 / 0	491 / 461
	Infected 10 parasites per 1 macrophage	Cell layer was destroyed				
	Infected 1 parasite** per 1 1 macrophage	16 / 57	30 / 34	52 / 9	2 / 0	302 / 681

* Expressed in mean intensity based on 6 detections in three different experiments
** Numerator indicates fluorescence intensity after quinacrine treatment before challenge, denominator shows intensity after quinacrine treatment 1 hr before examination

A few hours postchallenge the number of red granules (lysosomes) in macrophages with and without parasites as well as that of macrophages with a high content of red granules showed a slight decrease. After 24 hr in the cultures stained with quinacrine 1 hr before challenge (i.e. 25 hr before examination), the cells with a high red granule content were still few (Table I). In some parasite-containing macrophages, the number of fluorescing granules was perceptibly lower than that of phase-dense granules (Figs 6a and b). Similar preparations stained with quinacrine immediately before examination (i.e. 23 hr after challenge) contained generally more lysosomes per population than noninfected cultures (Table II). The fluorescent pictures of infected cells in such cultures corresponded to those obtained by phase-contrast microscopy (Figs 6c and d). This means that the loss of lysosomes at the time of challenge was subsequently compensated by the formation of new lysosomes and sometimes their numbers were excessive.

In all stages of cell cultures, single mesothelial cells and groups of them were observed in addition to macrophages. Toxoplasmas invariably penetrated in mesothelial cells and formed there colonies. Lysosomes, which are relatively evenly distributed in the cytoplasm of these cells, surrounded parasitophorous

Table II

*Changes in content of cells with different red granule content in population of macrophages after inoculation of supernatant of toxoplasma exudate**

Duration after inoculation hr	Macrophage cultures	Content of cells with different fluorescence intensity (conventional units) in macrophage culture (per cent)				
		High intensity	Medium intensity	Low intensity	Without granules	Mean intensity of 100 cells
4	Without inoculation	46	41	13	0	593
	Inoculated with supernatant 1 : 10	8	50	40	2	277
	Inoculated with supernatant 1 : 100	12	41	42	5	260
48	Without inoculation	25	45	30	0	413
	Inoculated with supernatant 1 : 10	38 53	33 36	29 11	0 0	508 649
	Inoculated with supernatant 1 : 100	22	48	30	0	395

* See footnote to Table I

vacuoles. We never observed the fluorescence of toxoplasmas nor of their lysosomes in mesothelial cells (Fig. 7).

Action of soluble products of toxoplasmas on macrophages. The washing- of the abdominal cavity of infected mice were actually a twice-diluted peritos neal exudate. After an intravenous injection of 0.50–0.20 ml, the supernatant of the washing killed mice within 1–2 min. This pointed to the presence of toxotoxin in the washings [14]. Supernatant, whether undiluted or diluted 1 : 10 or 1 : 100, which corresponded to infected exudate concentrations involved in the challenge with high and medium doses, was added to cultured macrophage medium. Lysosome content in macrophages and thus the number of cells with a high red granule content per population decreased early during incubation of supernatant-containing medium (Fig. 8, Table II). This decrease was more pronounced when the supernatant concentration increased. After 24 hr, the lysosome content of the preparations treated with fluorochrome before incubation differed slightly from the control level. Treatment with fluorochrome 1

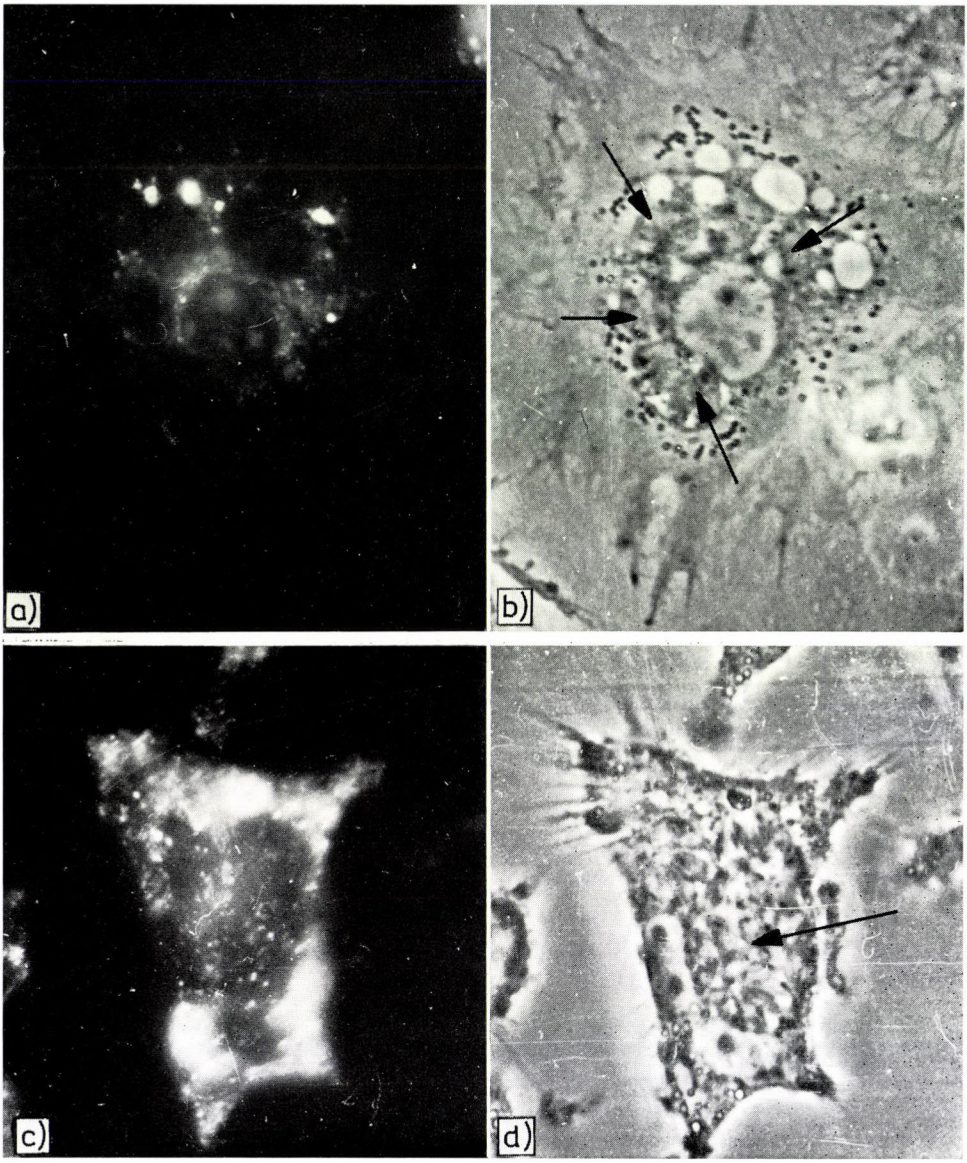

Fig. 6. Macrophages containing parasitic colonies (arrows) 24 hr postchallenge. In the cell fluorochrome-treated before challenge (a,b) the number of fluorescing granules (a) is lower than that of granules distinguishable by phase contrast (b). In the cell fluorochrome treated 1 hr before examination (c, d), the number of fluorescing granules (c) corresponds to that of phase dense ones (d). Toxoplasmas do not show fluorescence $\times 1000$

hr before examination (23 hr of incubation) was, however, followed by a considerable rise in the number of lysosomes in macrophages, as compared with the control (Fig. 9a and b).

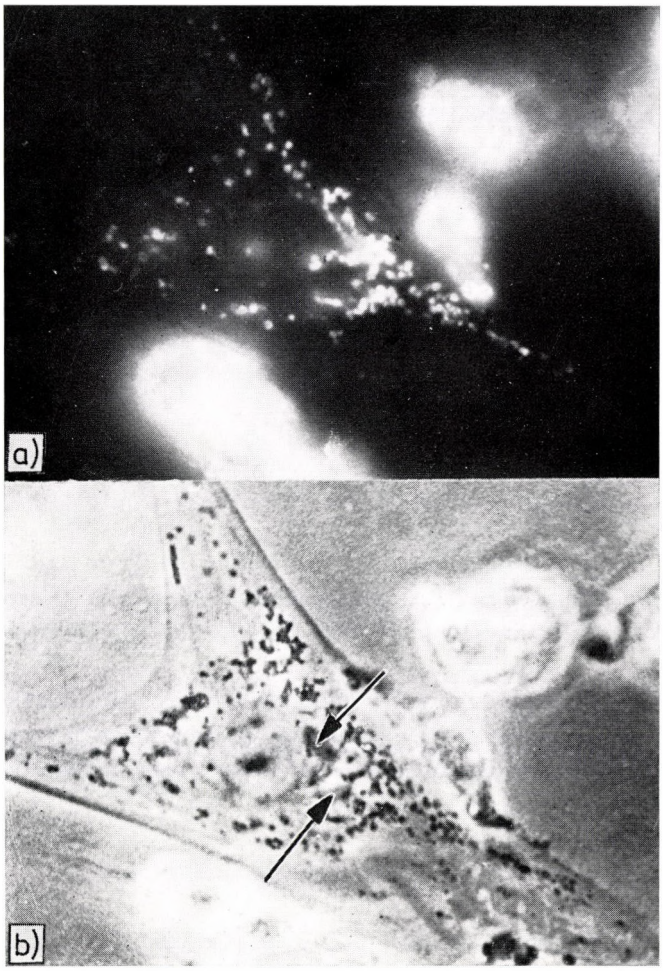

Fig. 7. Fluorescent (a) and phase-contrast (b) photomicrographs of the mesothelial cell in macrophage culture 24 hr postchallenge and 1 hr after staining with quinacrine. Fluorescence of lysosomes; toxoplasmas in the mesothelium (arrows) do not show fluorescence ×600

Acid phosphatase reaction. The results of this reaction were close to those of the fluorescence study. It was possible to distinguish 3 groups of cells in macrophage population in culture on the basis of the reaction rate. Table III shows that the fraction of cells with the highest enzymatic activity ($^{+++}$) in the macrophage population decreased 24 hr after low-dose challenge, the decrease being largely due to the diminished number of toxoplasma-containing cells. The decrease was particularly apparent at the stage of the large-scale degradation of infected cells, i.e. 48 hr after challenge with a low dose and 24 hr after challenge with a medium dose, and there was hardly any difference in the aspect of macrophages with and without parasites.

Table III

Changes in acid phosphatase reaction in population of macrophages after infection with toxoplasma

Duration of cell cultivation after infection hr	Cells in cultures	Content of cells (per cent) with different activity of acid phosphatase in population of macrophages after infection with					
		1 parasite per 1 macrophage			10 parasites per 1 macrophage		
		High activity	Medium activity	Low activity	High activity	Medium activity	Low activity
24	infected	31	55	14	12	70	18
	uninfected	52	38	10	14	78	8
48	infected	10	60	30	Cell layer was destroyed		
	uninfected	4	67	29			
Without infection		59	33	8	—	—	—

Control: infection with toxoplasmas of HeLa cells. Treatment with 2 μg/ml of quinacrine was followed by a selective red fluorescence of lysosomes, similar to that in macrophages and mesothelium. At a dose of 4×10^6 toxoplasmas per 1 ml, the entry and multiplication of parasites in HeLa cells proceeded as described elsewhere [15]. Fluorescence-phase contrast examination of the infected cell cultures treated with quinacrine showed that toxoplasmas inside parasitophorous vacuoles do not come in contact with the labelled content of lysosomes and do not fluoresce (Fig. 10).

Discussion

Quinacrine at a concentration of 2 μg/ml pinpoints lysosomes in all types of cells studied, toxoplasmas included. It may be supposed that this technique allows the identification of both primary and secondary lysosomes: (a) aminoacridine dyes penetrate into cells by diffusion rather than pino- or phagocytosis as thorotrast does [16]; (b) lysosomes are distinctly seen not only in macrophages and mesothelium but in HeLa cells, too. It is known that practically all lysosomes are secondary in those macrophages which reveal a high pinocytotic activity [5]. The mesothelium takes an active part in the vesicular transport of fluid [17]. Therefore, a considerable fraction of mesothelial lysosomes may contain exogenous material, too. It is the HeLa cells alone that are characterized by a low pinocytotic activity [18]. It may be supposed that many of their lysosomes are primary ones. It follows that toxoplasmas inhibit the fusion of both secondary [6] and primary lysosomes with phagosomes. This is further supported by the absence of fluorescence and, hence, the contact of viable toxoplasmas (i.e. those capable of forming colonies) with lysosomes.

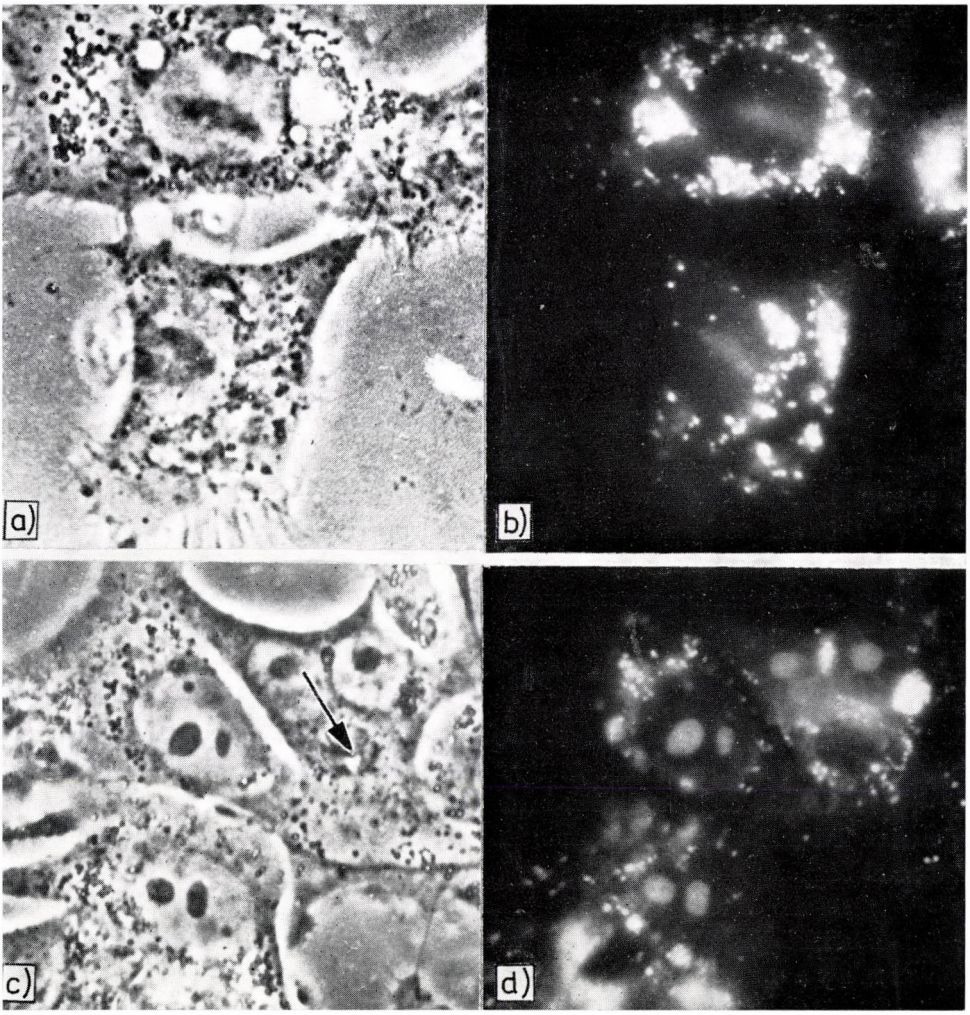

Fig. 10. Phase-contrast (a) and fluorescent (b) photomicrographs of the Hela cells, 24 hr post-challenge, 1 hr after staining with quinacrine. Lysosomes fluoresce red, the nucleoles of infected cells green. Toxoplasmas (arrows) are contained in the parasitophorous vacuole; they do not show fluorescence (d) and can be distinguished by phase-contrast (c) photograph only $\times 1000$

Therefore, toxoplasmas can inhibit the fusion of lysosomes with phagosomes in different types of cells and this ability is not limited to specialized phagocytes alone, but is an important factor of the pathogenecity of the parasite.

The fluorescence of some ingested toxoplasmas within a few hours after challenge indicates that they are non-viable and come in contact with the labelled content of lysosomes. If the fluorescence of lysosomes in toxoplasmas is considered to be a sign of their viability [10], the peritoneal exudate used in

this study for challenging is supposed to have contained 60% of viable parasites. This figure is close to the electron microscopic results obtained by JONES and HIRSCH [6].

The change of the fluorescence from green to red of phagocyted dead toxoplasmas points to a gradual accumulation of labelled contents in parasite-containing phagosomes. This change in fluorescence colour was observed in similar experiments with ingestion of yeasts by macrophages [19, 20]. The red fluorescence of dead parasites should not be interpreted as Strugger's effect, because a higher concentration of fluorochrome would be required to produce it. It is obvious from the report of JANSSEN [21] that high doses of fluorochrome should be used to produce Strugger's effect, when killed toxoplasmas are treated by acridine orange.

Highly infective doses of toxoplasmas inhibit parasite entry in macrophages and cause a considerable damage to cells, involving destruction of the cell layer. Medium and low doses cause a slight and reversible damage. Induction of these effects by means of a cell-free fraction of peritoneal exudate indicates that the actual damaging agent is one of the soluble products of toxoplasmas. The action of this agent is similar to that of some protein toxins of certain bacteria; it destroys the entire cell layer, non-infected cells included. This may be conveniently demonstrated with the acid phosphatase reaction at a time when infected macrophages begin to degrade and toxoplasmas and the products of their activity enter the nutrient medium. These data support the hypothesis that cells and tissues are damaged by the toxic products of toxoplasma activity [2–4]. It may be supposed that inhibition of pinocytosis by macrophages in the first 120 min after toxoplasma infection [22] is due to the toxicity of the infected suspension.

When the damage caused to macrophages by a low dose of toxoplasmas or their soluble products is slight, the decrease in the number of lysosomes is followed by a restoration of the normal level, or even an excessive output of cells. The latter phenomenon may be interpreted as a sign of mononuclear phagocyte activation, typical of toxoplasmic invasion [23]. The agent responsible for this activation is probably a soluble product of toxoplasmas. It is not clear whether agents which activate macrophages and those which damage cells, are identical. The study of their behaviour is a subject of further investigations.

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LIPID EXTRACTION AND PHOSPHOLIPID PHOSPHORUS DETERMINATION IN GRAM-POSITIVE BACTERIA*

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A comparison was made of the lipid yield of two modifications of the lipid extracting method of BLIGH and DYER and of that of GOULD and LENNARZ modified by us. Lipid extraction was done from 8 hr cultures of *Staphyloccus aureus* strain 5814S as a representative of Gram-positive bacteria. Extraction by the modified method of GOULD and LENNARZ resulted in an excess of 7.5–8.1 mg polar lipid per g dry weight of bacteria, compared to the yield of the two modifications of the method of BLIGH and DYER. The essence of our modification is that the enzymic activity and the further multiplication of bacteria is stopped by cooling the cultures rapidly to 6 °C with ice cones. After washing with ice cold buffer, the pellet is extracted in 5% trichloroacetic acid at 4 °C 2 times for 15 min in order to withdraw non-lipid materials that can be dissolved in lipid solvents. The extraction of lipids from the bacterial mass occurs in a mixture of water–methanol–chloroform 0.2 : 1 : 2 (v/v), pH 2.0, for two hours at room temperature. The extraction system is thoroughly shaken at quarter of an hour intervals. The separation of organic phases is performed by adding to the system 0.9% NaCl solution, pH 2.0. The separation becomes complete in half an hour if the system is left to stand after vigorous shaking. In the presence of Silica gel H, using 70% perchloric acid for phospholipid digestion, phosphorus determination proved to be of the same value as with the eluated phospholipid, i. e. in the absence of the gel. Phospholipid phosphorus estimation in the presence of Silica gel H took 1 or 2 days less, was less work, solvent and vessel-consuming, and the recovery of phospholipids was better.

Determination of lipid content in bacteria as well as in animal and human tissues or organs, and the study of the composition of lipids may be necessary for several diagnostic [1] and scientific purposes [2].

Cell walls of Gram-positive bacteria contain neither phospholipids nor neutral lipids in considerable amounts. Phospholipids are constituents of the plasma membranes and mesosomes, while the neutrals are mainly localised in the cytoplasm [3]. This circumstance is advantageous as the examination of phospholipids of such bacteria gives direct information on the phospholipid composition of membranes and mesosomes. In addition, it allows to combine such procedures for the complete extraction of lipids; in the case of Gram-negative bacteria they cannot be combined because of the presence of endotoxins.

Most of the methods for extracting bacterial lipids have been based on the procedure of BLIGH and DYER [4] described for the extraction of tissue lipids. The original method has been modified by several workers to allow complete extraction and to stabilize and simplify the procedure. In our labo-

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ratory a modification worked out by RÉDAI was used from 1967 to 1971 [5]. As the amount of lipids of *Staphylococcus aureus* extracted by this method seemed to be less than that published by other authors [6, 7], we decided to compare the lipid yield of the two modified methods of BLIGH and DYER [5, 7] with the lipid yield obtained by the modified method [8] of GOULD and LENNARZ [6] using the cultures of the same bacterial strain under the same conditions.

The bulk of lipids in any bacterium consists of phospholipids [2, 3, 5, 7–10]. The procedures for determining the quantity of its individual components are time-consuming and expensive. The essential steps are, purification of lipids by column chromatography, two-dimensional thin layer chromatography (TLC) on Silica gel, conversion of phospholipid phosphorus to inorganic phosphate and measuring with spectrophotometer [11–15]. The most work-consuming steps of lipid determination are the recovery of lipids by elution from the gel after the TLC, evaporation and drying.

Considering these difficulties, we decided to compare the phospholipid phosphorus determination performed with eluated lipids (i. e. in the absence of Silica gel) and with non-eluated lipids (i. e. in the presence of Silica gel) in order to omit the elution and the subsequent steps.

Materials and methods

Bacterial cultures. Two litres of an 8 hr culture of *S. aureus* 5814S as a representative of Gram-positive bacteria [8] were used for lipid extraction. The medium was a lipid-free Difco broth [16] and the conditions of cultivation were identical.

Lipid extraction. The compared lipid extracting methods are given in Table I. Essentially, ice cones of 400 ml volume were added to two litres of the culture to stop enzyme activity and growth. Washing was done at 4 °C. Non-lipid materials soluble in lipid solvents were extracted from the bacteria by 5% trichloroacetic acid (TCA) prior to lipid extraction. Each non-lipid extraction was performed at 4 °C for 15 min [17]. Lipid extraction was carried out at room temperature in a separatory funnel. At the end of the procedure the phases were fully separated by centrifugation. The upper phase was discarded and the lower chloroform phase containing the lipids was evaporated. On the next day the lipid extraction was repeated from the bacterial mass in the same manner. The lipids from the two extractions were united and then stored at −20 °C until further examination [18].

Thin-layer and column chromatography. Neutral lipids were separated from phospholipids on Silica gel G (Reanal) by one dimensional TLC according to SCHLIERF and WOOD [19]. For the elution of lipids from the gel the solvent systems described by WHITE and FRERMAN were used [7]. Lipids were purified from the Silica gel dust and from other non-lipid materials by column chromatography according to WELLS and DITTMER [15].

Determination of phospholipid phosphorus. After a previous comparison of a number of methods [11, 12, 14, 20, 21] and adaptation of certain parts of these methods to our circumstances, phospholipid phosphorus determination was carried out as follow. Separation of components of phospholipids was performed on neutral Silica gel H (Merck, Type 60) by two dimensional TLC [14]. In order to eliminate impurities from the Silica gel H it was washed in methanol-chloroform-formic acid 2 : 1 : 1 (v/v/v) prior to use [20]. Aliquots of the same amount (0.5 mg) of phospholipids were applied on the chromatoplates, then they were subjected to ascending prechromatography in the solvent system of acetone-petroleum ether 1 : 3 (v/v) to the top margin of the plate, to wash out impurities from the lipids and from the gel. The lipids remained on the plate [13]. Two-dimensional TLC took place first in chloroform-methanol-acetic acid 65 : 25 : 8 (v/v/v) [22] then, after drying, at right angles to the plate in chloroform-methanol-water 65 : 25 : 4 (v/v/v) [23]. Before visualisation of the components, phospho-

rus standards of 50, 75 and 100 nmole, respectively, were applied on the free area of the plates. To determine the total phosphorus content of the phospholipids, 0.1 mg of polar lipid was also applied. For the analysis of phospholipid phosphorus, a number of plates were exposed to iodine vapour and the spots were immediately outlined with the point of a needle. After the iodine had evaporated from the plate, each outlined spot was scraped off, and the phospholipid was eluted and then evaporated to dryness. On an other plate the phospholipids were visualised by ammonium molibdate spray [24].

After the components had become visible, each phospholipid spot and standard as well as the total phospholipid spot were outlined then scraped directly into a 20 ml calibrated pyrex test tube. Adjacent areas of blank Silica gel H corresponding in size and position to the areas containing phospholipid were also scraped into test tubes. Conversion of phospholipid phosphorus to inorganic phosphate was performed by 70% perchloric acid [12] in the presence of Silica gel H [20]. Phospholipids eluted from the gel were also digested. The further steps occurred according to Bartlett's phosphate determination [12]. After the colour had developed, the volumes of the samples were checked and made up with distilled water to the original volume. The suspensions were centrifuged to separate the fluid phase from the gel, then the extinction of the supernatants was read at 830 nm in a Jobin–Yvon spectrophotometer.

Prior to the phospholipid phosphorus determination the separated components were identified by their R_F values in several chromatographic systems [3, 7, 9, 10, 13, 14, 22, 23, 25]. Recently, phospholipid standards of Serva have been used.

Table I

Comparison of lipid extracting methods

Materials and procedures	Methods of		
	Bligh and Dyer [4], modified		Gould and Lennarz [6], modified by us
	by Rédai [5]	by White and Frerman [7]	
Inhibition of enzyme activity by	HCl pH4	HCl pH 2	ice, 6°C
Washing with	0.9% NaCl pH 5	buffer pH 7.1	buffer pH 7.2
Extraction of non-lipids by 5% TCA	once	—	two times
Lipid extraction			
Reflux in isopropanol ml	—	50	—
buffer, ml	—	60	—
water, ml	60	—	20
methanol, ml	150	50	100
chloroform, ml	60	75	200
pH	3	6.5	2
period, hr	2	2	2
buffer, ml	—	75	—
water, ml	60	—	—
chloroform, ml	60	75	—
0.9% NaCl, pH 2, ml	—	—	125
period, hr	1	1	0.5

Results

Lipid extraction. Table II gives a comparison of the lipid yield of the different lipid extracting procedures. The modifications of the method of BLIGH and DYER either by RÉDAI [5] or by WHITE and FRERMAN [7] resulted in essentially the same lipid yields (P = 0.5–0.6). On the other hand, our modification of the method of GOULD and LENNARZ seemed to be significantly more

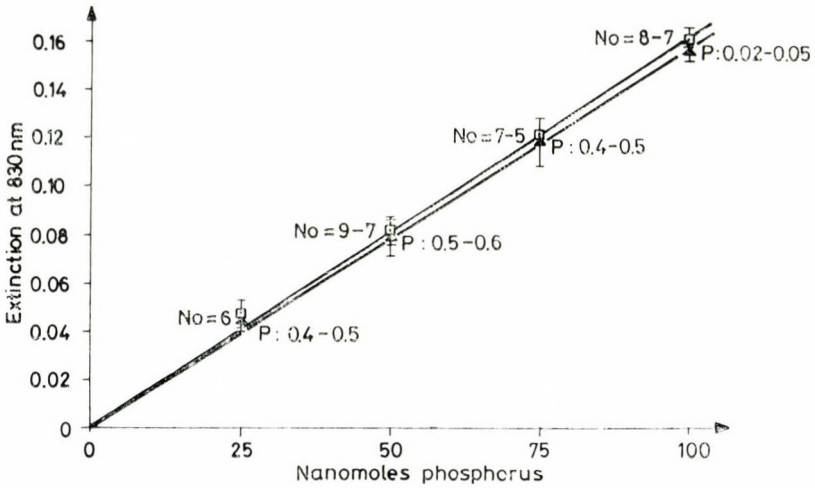

Fig. 1. Phosphorus standard curves without Silica gel and in the presence of Silica gel H. □——□ without Silica gel H; ▲——▲ with Silica gel H; No = number of measurements; P = probability of identity

favourable than the former two modifications. The lipid excess of 7.5–8.1 mg per g of dry bacteria, considering the deviations, derived exclusively from the better extraction of polar lipid ($P < 0.001$).

Determination of phospholipid phosphorus. Before comparing the procedures for phospholipid phosphorus determination, phosphorus standard curves were plotted with and without Silica gel H. Figure 1 shows the phosphorus standard curves.

As can be seen, below a 100 nmole phosphorus content the extinction values did not differ significantly in the two systems, indicating that the colour was not disturbed by the presence of Silica gel H. Further data are not given in Fig. 1 since over 100 nmole phosphorus the extinction did not proportionally increase with the phosphorus content in either system.

Next, the influence of Silica gel H on the digestion of phospholipids by perchloric acid, i. e. the conversion of phospholipid phosphorus into inorganic phosphate, and on the development of the colour was studied. The question was whether Silica gel H inhibited the digestion and the colour development.

Table II

Yield of lipids from eight-hour cultures of S. aureus 5814S by different extraction procedures

Method	No. of extractions	mg lipid per g of dry bacteria		
		total lipid	polar lipid	neutral lipid
I RÉDAI [5]	4	28.7±2.0	17.6±1.9	11.1±1.1
II WHITE and FRERMAN [7]	4	29.3±1.9	17.4±1.9	11.9±1.3
III GOULD and LENNARZ [6] modified by us	4	36.8±1.5	25.1±1.5	11.7±1.2
Differences between				
I and II		0.6	0.2	0.8
II and III		7.5	7.7	0.2
I and III		8.1	7.5	0.6

Results of these experiments are shown in Table III. It is seen that the lipid phosphorus values obtained in the presence of Silica gel H did not differ significantly from those obtained without Silica gel H. Without Silica gel H the deviations were greater than in its presence.

Table III

Comparison of phosphorus liberation from phospholipids by perchloric acid in the presence and in the absence of Silica gel H

Phospholipids (in 1 mg of polar lipid)*	No. of measurements	Nanomoles phosphorus			P
		in presence of Silica gel H	in absence of Silica gel H	difference	
Phosphatidic acid	6	80± 7	82±12	2	0.8–0.7
Phosphatidylglycerol	5	672±128	528±96	144	0.2–0.1
Lysophosphatidylglycerol	7	42± 4	48± 9	6	0.2–0.1
Diphosphatidylglycerol	6	89± 19	98±24	9	0.6–0.5
Lysylphosphatidylglycerol	5	91± 3	92± 5	1	0.8–0.7
C_{55}-isoprenylphosphate	5	47± 3	45± 3	2	0.3–0.2
Total	5	1023±133	899±100	124	0.2–0.1

* Extracted from 8 hour cultures of *S. aureus* 5814S by the procedure of *Gould* and *Lennarz* (6) modified by us.

Discussion

We have modified and simplified the lipid extracting procedure of GOULD and LENNARZ [6]. The lipid yield of the modified method proved significantly greater than that of the modified methods of BLIGH and DYER [5, 7]. Besides, the procedure developed by us has a number of advantages. The greater yield of lipids derives probably from several factors of which the most important are the following. Treatment with TCA before lipid extraction results in a withdrawal of non-lipid materials that can be dissolved in lipid solvents. Consequently, part of the solvents remains for lipids. Furthermore, TCA treatment loosens the structure of bacteria. The proper pH value of the extraction system seems to be essential. The best mixture of solvents is obtained at pH 2.0. In the first system, the 2 to 1 ratio of chloroform and methanol increases the solving capacity of the system to polar lipids. In the second system the large volume of salt solution accelerates separation of the organic phases and the concentration of lipids into the chloroform phase. During one half hour standing the phases become separated so perfectly that centrifugation may be omitted. Keeping the pH of the systems at 2 results in an inhibition of the activity of phospholipases.

The yield of C_{55}-isoprenylphosphate can considerably be increased if the mass of bacteria is subjected to further extraction in n-butanol and 6 M pyridine acetate 4 : 1 (v/v) [9] or a 2 : 1 mixture of these solvents [26].

Determination of phospholipid phosphorus in the presence of Silica gel H takes 1–2 days less and is cheaper as the phospholipids are first eluted from the gel. At the same time, the loss of lipids is also less. In the case of those components that give diffuse spots on TLC, e.g. phosphatidylglycerol, the edges of the spots can be visualized by ammonium molybdate spray [24] better than by iodine vapour, so it is easier to outline the spots after spraying.

When digestion by perchloric acid takes place, attention must be focussed at ensuring a perfect suspension of Silica gel H in the reaction system. For this very reason, the volume of perchloric acid solution must be increased according to the amount of gel in the tube. On the other hand, when the amount of the gel is small, the volume of the digesting solution may be decreased. Of course, changes in the volumes have to be considered when calculating the phosphorus content. Before starting the digestion, it is advisable to mix the reaction system thoroughly but carefully.

A number of methods that use sulphuric acid for digestion of phospholipids [11, 20, 21] was also tested. These procedures are more complicated than is the perchloric acid digestion. Moreover, phosphorus standards made up with sulphuric acid of different firms except Merck, gave a line that started above zero. In other words, the blanks contained some phosphorus, consequently they exhibited some extinction.

Our results indicate that for lipid extraction from Gram-positive bacteria it is advisable to utilize our modified method of GOULD and LENNARZ. On the other hand, conversion of phospholipid phosphorus into inorganic phosphate by perchloric acid digestion by Bartlett's procedure in the presence of Silica gel H is a fast, cheap and reliable method [8].

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EFFECT OF METHICILLIN ON THE PHOSPHOLIPID CONTENT OF METHICILLIN SENSITIVE STAPHYLOCOCCUS AUREUS*

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The effects of methicillin at concentrations of 1 μg/ml (= ½ × minimum inhibitory concentration) and 2 μg/ml (= 1 × MIC) on the phospholipid content of methicillin-sensitive *Staphylococcus aureus* 5814S growing exponentially were studied. During 2-hour treatment ½ × MIC of methicillin induced a significant increase in the phospholipid content of the cocci; accumulation of acid phospholipids was prevalent. In contrast, addition of 1 × MIC of methicillin to the cultures resulted in significantly less phospholipids in the cocci compared with that of the control cultures. There were some alterations in the amounts of individual phospholipids during treatment. The phosphatidic acid (PA) and diphosphatidylglycerol–cardiolipin (DPG) contents were significantly reduced in the first hour then reached the control values by the end of the 2nd hr of exposure. An opposite change was found in the amount of lysylphosphatidylglycerol (LPG), whereas the phosphatidylglycerol (PG) level was significantly lower in the treated cultures than in the controls of corresponding age. Phospholipid synthesis seems to be one of the sites of the action of methicillin in *S. aureus*.

Cells of *Staphylococcus aureus* grown in the presence of subinhibitory concentrations of methicillin show irregularities in shape, size and staining. Large swollen cocci were seen on films [1]. Our electron microscopic observations and morphometric studies revealed that growth of the methicillin-sensitive cells of *S. aureus* strain 5814S in the presence of 0.5 μg methicillin per ml for 14 hr resulted in a significant increase in both cytoplasmic and whole cell volume [2, 3].

The effect of methicillin on the permeability of *S. aureus* 524SC was measured by Rogers who found a maximum increase of permeability to 8-anilino-1-naphthalene sulphonic acid at 2 μg/ml, and the same concentration produced the highest rate of death and lysis [4]. In our experiments, cells of *S. aureus* 5814S which had grown in the presence of methicillin (0.25 μg/ml) for 18 hr showed considerable changes in the composition of their phospholipids [5]. The results of these experiments indicate that exposure to the antibiotic of methicillin-sensitive cocci renders them osmotically fragile.

It is known that the cell wall plays a passive role in cell permeability [6–8] while the cellular element responsible for the maintenance and regulation of normal transport is the cell membrane. Moreover, there is evidence that enzymes involved in wall synthesis are localized in the membrane and mesosomes

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[8–13], and the activity of some of the wall synthetizing enzymes are lipid-dependent [11, 14], and the lipids required for enzyme activity are essential structural and functioning constituents of the membrane and mesosomes [6, 7, 14–19].

Considering the structure and role of the membrane in cell integrity, we have assumed that the inhibition of lipid synthesis by methicillin is involved in the killing effect of the antibiotic, in other words that phospholipid synthesis may be a candidate for methicillin killing sites. If lipid synthesis is involved in the cell killing sites of methicillin, the cells unable to survive under the action of the antibiotic will contain less lipids than those capable of surviving the effect of methicillin [5, 20].

In this paper we describe the effect of methicillin at concentrations of $\frac{1}{2} \times$ MIC and $1 \times$ MIC on the phospholipid content of cells of the methicillin-sensitive strain *S. aureus* 5814S growing exponentially.

Materials and methods

Strain. All the experiments were carried out with the methicillin-sensitive strain *S. aureus* 5814S [21].

Medium. Cells were grown in lipid-free Difco-broth containing 1% glucose pH 7.2, in which all constituents except casein hydrolysate were defined chemically [22].

Antibiotic. Methicillin was purchased from Chinoin, Budapest.

Cultivation. In ten-litre retorts 7 litres of broth were inoculated with an appropriate dilution of 18 hr cultures of the strain, so that the final inoculum contained $1.2–1.5 \times 10^7$ organisms per ml. Incubation was done at 37°C. The cultures were stirred with Teflon-covered magnetic stirrers for 5 min at half hour intervals. When the extinction of the cultures had reached the value of 0.65, equivalent to $5–7 \times 10^8$ bacteria per ml, determined with a Spectromom 202 spectrophotometer at 650 nm, methicillin was added at concentrations indicated in the Figures. At 0, 1 and 2 hr later samples were taken for determining the viable counts and dry weight, and for the extraction of lipids.

Lipid extraction. A modified method of GOULD and LENNARZ [17] was used for the extraction of total lipid [3, 23, 24]. C_{55}-isoprenylphosphate (C_{55}-IP) was extracted by the procedure of ANDERSON *et al.* [25]. Non-lipids were removed on Sephadex G-25 fine column (Pharmacia) as described by WELLS and DITTMER [26]. Polar lipids were separated from neutrals by one-dimensional thin layer chromatography (TLC) on Silica gel-G plates (Merck) in the developing system of SCHLIERF and WOOD [27].

Determination of phospholipid phosphorus. Separation of the phospholipid components was performed on purified neutral Silica gel-H plates (Merck) by two-dimensional TLC [28], developing first in chloroform-methanol-acetic acid, 65 : 25 : 8, then in chloroform-methanol-water, 65 : 25 : 4 [29]. Phospholipids were visualized by spraying with ammonium molybdate-perchloric acid [30]. The spots were removed into tubes and phospholipid phosphorus was converted into inorganic phosphate by 70% perchloric acid in the presence of Silica gel-H [3, 23, 24]. Estimation of phosphate was carried out according to Bartlett [31].

Results

Figure 1 shows the effect of methicillin on the viable counts in cultures of *S. aureus* 5814S.

Prior to the addition of methicillin, the cells were growing exponentially. After adding 1 μg/ml ($= \frac{1}{2} \times$MIC) to the cultures, multiplication continued

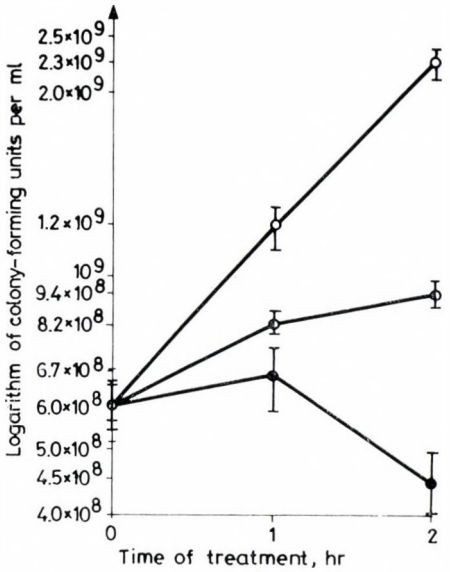

Fig. 1. Semilogarithmic representation of the effect of methicillin on the growth kinetics in the exponential phase of methicillin-sensitive *S. aureus* 5814S. ○—○ control (6); ◐—◐ 1μg methicillin per ml = ½×MIC (5); ●—● 2μg methicillin per ml = 1×MIC (5); figures in parentheses indicate the number of experiments

and a significant gain in viable count occurred in the first hour of methicillin exposure. Between the first and second hours of treatment, the multiplication failed to proceed but there was no decrease in viability. Thus, under the influence of ½×MIC of methicillin the majority of the population was capable of survival and of doubling for a certain period of time but the cells multiplied at a significantly lower rate. Exposure of the exponentially growing cells to 2 μg/ml (1×MIC) inhibited further multiplication and caused a decrease in viable count for a period of 2 hr.

The changes in optical density of the cultures treated with methicillin at various concentrations were measured simultaneously with the determination of the viable counts by plating. Figure 2 demonstrates the effect of methicillin on optical density.

The results were somewhat surprising. The optical density of the cultures treated with ½×MIC of methicillin increased roughly parallel with the viable counts. The optical density of the cultures exposed to 1×MIC of methicillin increased, however, during the treatment while the viable counts decreased significantly (see Fig. 1). This contradiction might derive from two facts. On the one hand, many cells were already dead but not lysed, therefore the total number of cocci was much higher than the number of the surviving ones. On the other hand, when thin sections were made of the cells from the cultures treated with 1×MIC of methicillin, electron microscopy revealed granulation

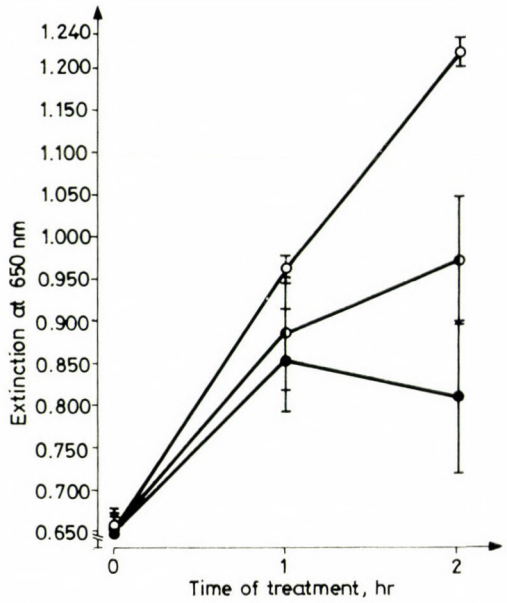

Fig. 2. Changes in the optical density of exponentially growing cultures of *S. aureus* 5814S treated with methicillin. ○—○ control (6); ◐—◐ 1 μg methicillin per ml = ½×MIC (5); ●—● 2 μg methicillin per ml = 1×MIC (5)

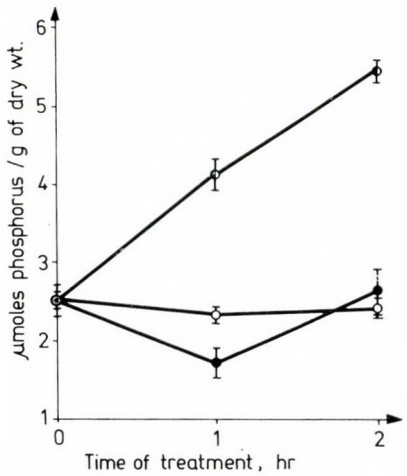

Fig. 3. Changes in the phosphatidic acid (PA) content of exponentially growing *S. aureus* 5814S treated with methicillin. ○—○ control (4, 12); ◐—◐ 1 μg methicillin per ml = ½×MIC (5, 15); ●—● 2 μg methicillin per ml = 1×MIC (5, 15)

and then an aggregation of granules in the cytoplasm of many cells. A few cocci were only lysed, and contamination of the bacterial pellets by membrane elements was poor [3]. Thus, the discrepancy between the viable counts and the optical density of the cultures treated with $1 \times$MIC of methicillin was partly due to the dead cocci and partly to the aggregation. The dry weight of the cultures corresponded with the optical density.

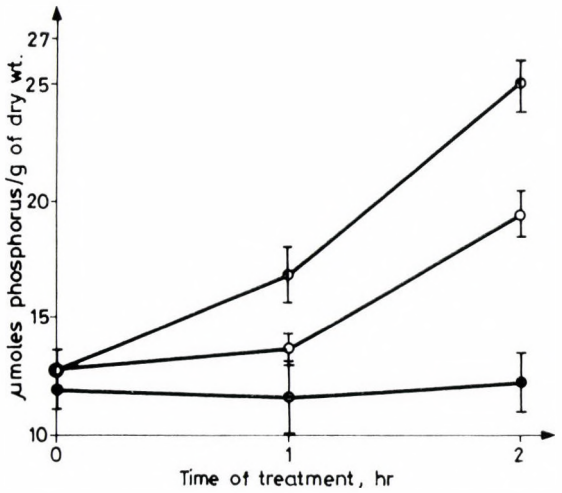

Fig. 4. Changes in the phosphatidylglycerol (PG) content of logarithmically growing *S. aureus* 5814S treated with methicillin. ○—○ control (4, 12); ◐—◐ 1 μg methicillin per ml = $\frac{1}{2} \times$MIC (5, 15); ●—● 2 μg methicillin per ml = $1 \times$MIC (5,15)

Figure 3 shows the effect of two concentrations of methicillin on the PA content of the cocci during a period of 2 hr. The 1st figure in parenthesis indicates the number of experiments and the 2nd figure indicates the number of measurements. It can be seen that the PA level of the control cells did not change during the long-phase examined. Addition of $1/2 \times$MIC of methicillin to the cultures caused a continuous and significant increase in the PA content. In contrast, under the influence of $1 \times$MIC of methicillin, the PA content of the cells fell rapidly in the first hour then returned to the control value by the end of the second hour.

Figure 4 shows the changes in the PG content of cocci treated with methicillin. PG was the major component of the phospholipids in *S. aureus* 5814S. Since PG had been shown to catalyse the activity of translocase, an enzyme involved in peptidoglycan synthesis by *S. aureus* [11], it seemed of interest to measure the PG level of the methicillin-treated cells. Figure 4 indicates that the PG content of the control cells increased with the duration of the log-phase. Exposure of the cultures to $\frac{1}{2} \times$MIC of methicillin brought about a near 2-fold

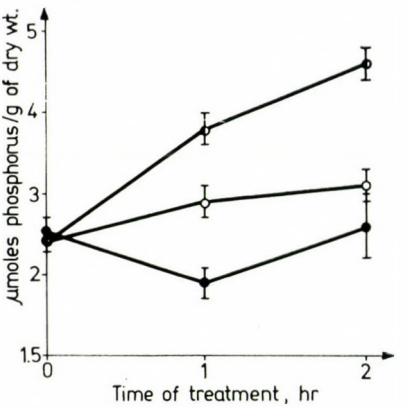

Fig. 5. Changes in the cardiolipin (DPG) content of *S. aureus* 5814S growing exponentially exposed to methicillin. ○—○ control (4, 12); ◐—◐ 1 μg methicillin per ml = ½×MIC (5, 15); ●—● 2 μg methicillin per ml = 1×MIC (5, 15)

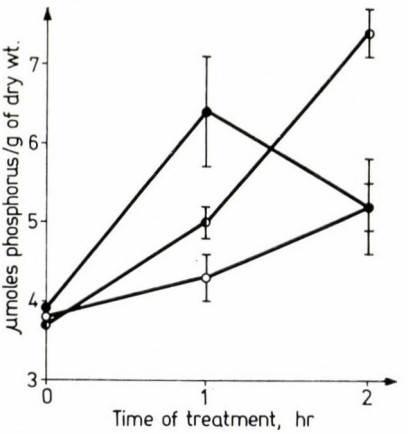

Fig. 6. Changes in the lysylphosphatidylglycerol (LPG) content of *S. aureus* 5814S growing exponentially exposed to methicillin. ○—○ control (4, 12); ◐—◐ 1 μg methicillin per ml = ½×MIC (5, 15); ●—● 2 μg methicillin per ml = 1×MIC (5, 15)

increase in the PG content of the cells. The increase was significantly higher than in the controls. In contrast, the addition of 1×MIC of methicillin to the cultures inhibited the synthesis of PG or enhanced greatly its hydrolysis. In fact, after growth with 2 μg/ml for 2 hr, the PG level was as low as it had been at 0-time, and differed significantly from the control level.

Figure 5 demonstrates the effect of methicillin on the DPG content of the exponentially growing cocci. In the control cultures there was a slight increase in the amount of DPG during the log-phase. Under the effect of ½× MIC of methicillin a significant accumulation of DPG occurred in the cells,

while 1×MIC caused a significant decrease in the DPG content of the cells in the first hour of exposure, then it increased to the 0-time value.

Figure 6 shows the LPG content of the cells exposed to methicillin. LPG is one of the most important phospholipids since takes part in a number of metabolic processes [32—34]. As can be seen from Fig. 6, the LPG level of control

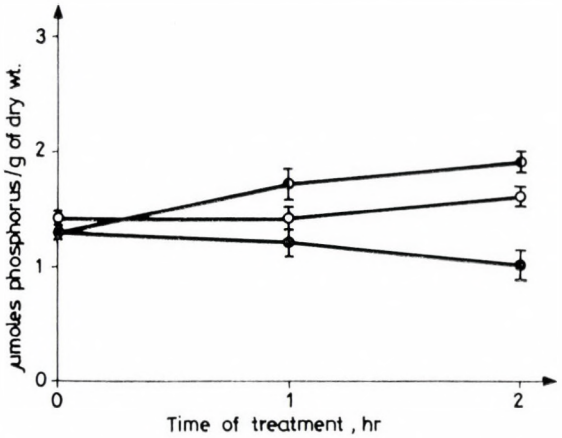

Fig. 7. Effect of methicillin on the bound C_{55}isoprenylphosphate (C_{55}-IP) content in *S. aureus* 5814S growing exponentially. ○—○ control (4, 12); ◐—◐ 1 μg methicillin per ml = ½×MIC (5, 15); ●—● 2 μg methicillin per ml = 1×MIC (5, 15)

cells increased parallel with the incubation time. The addition of ½×MIC of methicillin induced a significant accumulation of LPG in the cocci.

In contrast to the other phospholipids, under the influence of 1×MIC of methicillin the LPG level did not decrease, but was significantly enhanced by the cells in the first hour of the treatment then it fell to the control value.

Figure 7 demonstrates the effect of methicillin on the C_{55}-IP content of exponentially growing cocci. C_{55}-IP is known to be a carrier in the peptidoglycan synthesis of *S. aureus* [25]. The control cells contained the same amount of C_{55}-IP during the course of the log-phase studied. Half the MIC of methicillin increased significantly, while 1×MIC reduced significantly, the amount of C_{55}-IP in the cells.

Figure 8 shows the amount of total phospholipid in cells treated with methicillin. The structure and functions of the lipid part of the staphylococcal membrane are considerably determined by integrating the individual phospholipids to total phospholipid. Figure 8 shows that the total phospholipid content of the control cells increased significantly during the log-phase studied. Under the influence of ½×MIC of methicillin, total phospholipid synthesis was stimulated or phospholipid catabolism was inhibited resulting in a significantly larger amount than that found in untreated cells. In contrast, ad-

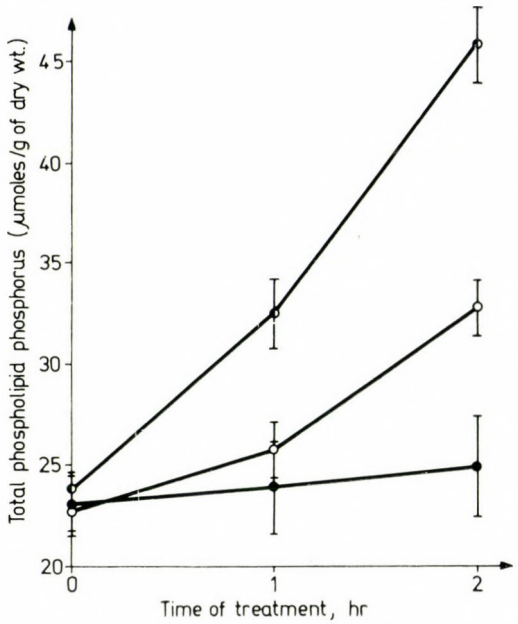

Fig. 8. Effect of methicillin on the phospholipid content of S. *aureus* 5814S growing exponentially ○—○ control (4, 12); ◐—◐ 1 μg methicillin per ml = ½ × MIC (5, 15); ●—● 2 μg methicillin per ml = 1 × MIC (5, 15)

dition of 1 × MIC of methicillin to the cultures seemed to stop the further synthesis of phospholipids. In fact, the amount of total phospholipid in the cells treated with 1 × MIC of methicillin for 2 hr was similar as the 0-time value, and significantly less than that in the controls or in cells exposed to ½ × MIC of the antibiotic.

Discussion

The response of lipid metabolism in sensitive staphylococcal cells to antibiotic treatment was unknown before 1971. Since then a number of antibiotics has been shown to influence lipid synthesis and metabolism, in dependence on the concentration and the kind of antibiotic [5, 35, 36]. Our results are in accordance with those obtained with antibiotics interfering with cell wall synthesis.

The most surprising fact is that methicillin strongly influences the phospholipid content of sensitive staphylococcal cells growing exponentially. Lipid synthesis seems to be very sensitive to the action of methicillin, the effect of which on the lipid content of cells greatly depends on its concentration. When treatment occurs with 1 μg/ml (= ½ × MIC), the majority of cells has the possibility to survive, and in these cells the amount of phospholipids increases

significantly. Lipid accumulation originates mainly from increase in acid-phospholipids. To increase the amount of lipids by the surviving cells may be advantageous in several respects. It increases the compactness of the membrane structure. The excess of lipid increases the impermeability to water by increasing the hydrophobic nature of the membrane. Furthermore, it increases the activity of enzymes that require phospholipids [10, 11, 14].

The results raise the problem of lipids in the survival of staphylococcal cells after exposure to $\frac{1}{2} \times$ MIC of methicillin. The role of lipids is supported by the physico-chemical studies on the interaction of phospholipids with penicillins resulting in various decreases in the effectiveness of penicillins [37, 38].

On the other hand, when cells are treated with 2 μg/ml ($1 \times$ MIC) of methicillin inhibiting their growth abruptly, an almost complete inhibition of the synthesis of acid phospholipids is observed. Fast catabolism of these lipids is unlikely to take place but this possibility cannot be excluded. In any case, methicillin at growth inhibitory concentrations appears to exert a dramatic effect on cells by preventing the necessary increase in the amount of acid phospholipids, some of which are activators of enzymes. Besides, lowering of the lipid content in the membrane is accompanied by an increase in the permeability to water due to the fall in hydrophobicity. This range of events with the increased activity of wall hydrolytic enzymes [4] may lead to the death of cells by disruption in the presence of methicillin in growth inhibitory concentrations.

Until now, methicillin and the other beta-lactam antibiotics have been known to act only on the cell wall synthesis of bacteria [9, 13, 39, 40]. Our results allow to conclude that methicillin has at least two sites of action in *S. aureus*. One is peptidoglycan synthesis and the other is phospholipid synthesis. As far as we are aware, the earliest findings concerning the effect of methicillin on phospholipid synthesis in *S. aureus* came from our laboratory [3, 5, 20].

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LYSOSOMAL ACRIDINE ORANGE UPTAKE IN FIBROBLASTS TRANSFORMED BY SV40 or HUMAN CYTOMEGALOVIRUS*

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Lysosomes of living human fibroblasts, SV40-transformed rat fibroblasts and human CMV-transformed hamster fibroblasts were examined by fluorescene microscopy after pretreatment with acridine orange at a supravital concentration (5×10^6 M). Dye uptake by human primary fibroblast lysosomes was considerable and independent of the age of the cultures. In the transformed cultures, cytoplasmic granular red fluorescence indicating lysosomal acridine orange uptake could not be observed in part of the cells; cells showing no cytoplasmic granular fluorescence appeared as early as after 48 hr incubation and were growing in dependence on the age of the culture. Staining of living cells by acridine orange solutions at supravital concentration is a practicable method for the examination of functional changes of lysosomes.

ZELENIN and his coworkers [1–3] were the first to identify with lysosomes the particles fluorescing in a bright red colour in the cytoplasm of living cells pretreated with an acridine orange solution of supravital concentration. Acridine orange (2,8-bis-dimethylamine acridine) monomers and polymers fluoresce in green and red colour, respectively. Amino-acridines show a well-defined affinity for lysosomes. When their concentration in the lysosomes has reached a certain level, polymerization results in the appearance of red fluorescent granules in the cytoplasm.

It was proved soon after the discovery of lysosomes that lysosomal enzymes play an important part in inducing cell division. Similar observations were made in livers regenerating after partial hepatectomy [4].

It is well-known that stabilizers of the lysosome membrane inhibit cell division. It is also obvious that lysosomal enzymes are continuously or gradually released by tumour cells. Still, the role of lysosomal enzymes in the continuous proliferation of tumour cells has not been clarified satisfactorily. SYLVEN [5], having examined the enzyme contents of interstitial tumour fluids, concluded that lysosomal enzymes flow into the extracellular space from tumour cells and from cells of the tumour-bearing organism. CAREVIC *et al.* [6] have shown that acid phosphatase is released from cells in livers infiltrated by leukaemia cells. According to URBAN and UNSWORT [7] the lysosomal acid

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phosphatase activity of Novikoff hepatoma cells and of cells of animals bearing the tumour is diminished and the lysosomes are decreased in number.

In the present report fluorescence-microscopic lysosomal changes observed in cell lines cultivated *in vitro* and transformed by DNA viruses are described.

Materials and methods

Cell cultures. (i) Primary human fibroblast cultures; (ii) the rat fibroblast SV_{68} C—3Y1—1 subclone [8] transformed by a wild strain of simian virus 40 (SV40); (iii) TRH clone originating in hamster fibroblasts transformed by human cytomegalovirus (CMV; (iv) HEp-2 established cell line.

Cells were cultivated on coverslips in Dulbecco's nutrient fluid containing 10% bovine serum. After incubation for 24, 48, 72, 96 and 120 hr, coverslips were transferred into a maintenance fluid containing 5×10^{-6} M acridine orange, but no serum, and incubated for 15 min at room temperature. The coverslip cultures thus prepared were washed in PBS, then placed on a slide and examined in an Opton Universal Fluorescence Microscope at 390 nm wave length. Photographs were taken on Agfacolor film with 1 to 2 min exposure time. The numerical decrease of cells eliciting granular fluorescence were examined in coverslip cultures in each of which 100 cells were counted.

Results

The cells washed in PBS stained well with 5×10^{-6}M acridine orange. The little stain remaining in the interstitial substance gives no fluorescence, thus the microscopic picture is sharp and can well be photographed. It is also easy to differentiate living cells from dead ones because the latter have a diffuse red fluorescence. The lysosomes of the living cells, on the other hand, show a granular red fluorescence; the granules are accumulated mainly around the nucleus and only scattered lysosomes occur elsewhere in the cytoplasm.

Bright red granular fluorescence was visible in every living human fibroblast, regardless of the age of the culture; a 120 hr culture is shown in Fig. 1.

Every SV40 transformed rat fibroblast showed red fluorescence in the 24 hr cultures (Fig. 2), whereas in 48 hr cultures some non-dividing cells were devoid of red-fluorescing granules, while others contained just a few (Figs 3 and 4). During prolonged cultivation non-fluorescing cells were increasing in percentage. The relationship between the decrease of lysosomal fluorescence and the age of the culture is illustrated in Fig. 5.

The cells showing cytoplasmic diffuse green fluorescence and well-visible nucleoli may be regarded as living cells even if no granular red fluorescence can be detected in them.

The morphological changes observed in cultures of hamster fibroblasts transformed by human CMV were similar to those observed in the SV40-transformed cell cultures.

Next, lysosomal acridine-orange uptake of the established cell line HEp-2 was examined. Only a proportion of the cells displayed a granular red fluores-

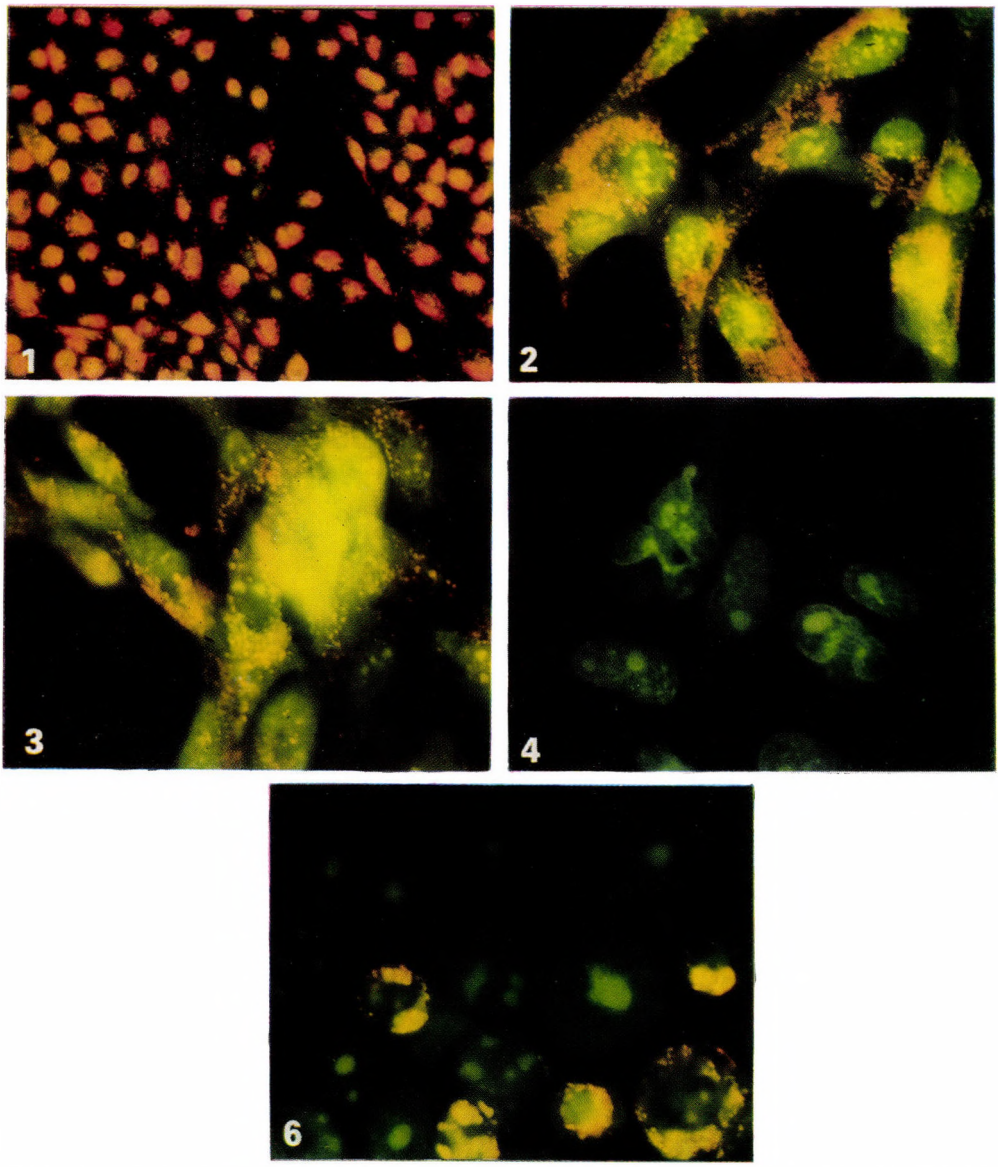

Fig. 1. Human fibroblasts in 120 hr primary culture. Acridine orange stain (5×10^{-6} M). Agfacolor film, exposure time 1 min $\times 250$

Fig. 2. Twenty-four-hour culture of SV40-transformed rat fibroblasts. Exposure 2 min $\times 400$

Figs 3 and 4. Forty-eight-hour culture of SV40-transformed rat fibroblasts. Exposure time 2 min $\times 400$

Fig. 6. Cells from a 72-hr culture of HEp-2 cells. The cells were cultivated in flasks, then trypsinized and the cell suspension thus obtained was treated with acridine orange. Exposure time 2 min $\times 400$

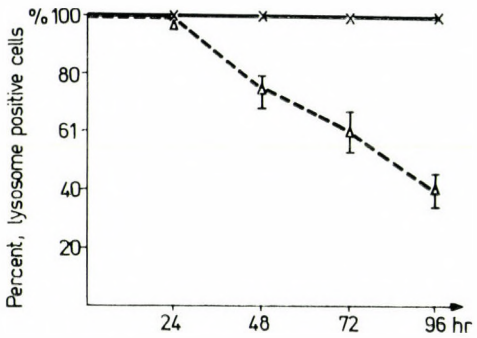

Fig. 5. Age-dependent changes in the number of cells containing demonstrable lysosomes during incubation; × human fibroblast cells; △ rat fibroblasts cells transformed by SV40

cence after the above treatment. Figure 6 shows a 72 hr culture of HEp-2 cells.

The distribution of cells giving lysosomal fluorescence in different 5- day cultures is presented in Table I.

Table I

Lysosomal acridine orange uptake by primary human fibroblasts and cultured tumour cells

Cultured cells	No. of experiments	Positive cells in 5-day cultures, per cent
Primary human fibroblasts	6	100
Rat fibroblasts line transformed by SV40	6	38 ± 12
Hamster fibroblast line transformed by human CMV	5	43 ± 9
Ehrlich ascites tumour cells	4	0

Using the same technique we failed to observe granular red fluorescence in Ehrlich ascites cells of the mouse.

Discussion

It has been known since the publications of ZELENIN *et al.* [1–3] that the lysosomes of living cells take up acridine orange if the dye is applied at supravital concentration (10^{-5}–10^{-6} M). The dye uptake is the result of an active cell function. Polymerization of the dye molecules requires no active cell function. Aggregation and polymerization of dye dipoles is assumed to occur with

the co-operation of water molecules, if the distance between neighbouring dipoles is less than 0.5 nm. The relationship between the degree of polymerization of fluorochromes and their colour has been discussed by MÓDIS [9].

The lack of lysosomal granular red fluorescence in some tumour cells may have two causes, an inhibition of active uptake or else the diminished enzyme content of lysosomes may not be sufficient for acridine orange polymerization. We prefer the latter alternative because *in vitro* every young tumour cell, except the dividing ones, displays a granular red fluorescence. In 48 hr cultures the outflow of enzymes from the tumour cell lysosomes may be so intensive that the dye molecules that have been taken up cannot be polymerized in the lysosomes. This assumption has been supported by several investigators. NICHOLSON [10], examining the enzyme content of dimethylbenzanthracene-induced rat mammary tumours, found an increase in lysosomal enzyme activity during the regression following hormone treatment. KASUKABE *et al.* [11] reported that glucocorticoid treatment of Ml myelogenous leukaemia cells of SL mouse strain origin was followed by cell differentiation and that during this differentiation lysosomal enzymes were induced.

From our own experiments and literary data we therefore assume that the lysosomes of tumour cells function differently from the lysosomes of normal cells, in that enzymes are continuously released from tumour cell lysosomes. The process may be necessary for averting contact inhibition.

The lysosomal changes in living cells can easily be followed by fluorescence microscopy after staining with acridine orange at supravital concentration.

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IN VITRO TRANSFER OF MULTIPLE RESISTANCE OBSERVED IN VIVO DURING A SALMONELLA LONDON EPIDEMIC

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Between 1976 and 1978, waves of *Salmonella london* infections conveyed by raw meat and meat products were observed. The strains isolated during the epidemic were first susceptible then developed multiple antibiotic resistance. The identical antibiotic resistance patterns of the strain and their more frequent occurrence in hospital environments indicated plasmid-mediated resistance. R-plasmid transfer, minimum inhibition concentration and resistance elimination were studied in representative strains. The resistant *S. london* strain and transconjugants of *Escherichia coli* rendered resistant were compared. The results proved that multiple resistance was plasmid-mediated.

In the past 15 years a large body of laboratory and epidemiological evidence has accumulated on the spread of multiresistant bacteria in hospital environment. It has been demonstrated that antibiotic resistance of the strains is linked to the DNA sequences carried by the plasmids. The recently recognized transposons [1] possess the capacity to move between the plasmids, therefore it can be assumed that they play an important part in the development of multiple resistance. Bacteria bearing R plasmids are permanent potential donors of the carried resistance. Transfer of R plasmids from one enteric bacterium to the other can easily be assessed in experiments *in vitro*. It is more difficult to prove that transmission has occurred *in vivo*. Usually, only a subsequent analysis of the strains isolated during the outbreak [2], the *in vitro* tests and the clarification of the epidemiologic background can verify *in vivo* transfer [3]. It is because of these difficulties that usually no examinations are made to demonstrate whether a patient excreting or carrying the multiple resistant strain did excrete or carry any apathogenic enteric bacteria serving as a vector for multiple resistance.

The present study was done (i) to analyze the changes in antibiotic resistance of the strains in view of the epidemiological and food bacteriological background; (ii) to test the transferability of the R plasmid of the multiresistant strains; and (iii) to investigate resistance transfer by genetic techniques in order to supplement the epidemiological observations.

Materials and methods

Strains. Three representative strains were selected from those isolated during the epidemic. *Salmonella london* 1207 was isolated in the course of environmental screening of foodstuffs, strains *S. london* 11249 and 12641 were isolated from the stool samples of patients with enteritis. As recipient strain, *Escherichia coli* K12 Hfr lac$^-$ nal^r was used.

Culture media. Infusion broth: Bacto peptone (Difco), 1 g; Lab-Lemco beef extract (Oxoid), 10 g; NaCl, 5 g; distilled water, 1000 ml; pH 7.4; 121°C 30 min. The solid medium was prepared by adding 11 g of Oxoid agar No. 1 per 1000 ml broth. The antibiotic resistant transconjugants were selected on eosin methylene blue agar, to which tetracycline or kanamycin (50 μg/ml) or ampicillin or chloramphenicol (25 μg/ml) was given. Nalidixic acid (50 μg/ml) was incorporated in all media supplemented with antibiotics.

Determination of antibiotic resistance. Resistest disks (Institute for Serobacteriological Production and Research, Human, Budapest) were used.

Determination of minimum inhibition concentration (MIC). The tube dilution technique was applied using approximately 10^5 cell inocula. The cultures were incubaed at 37°C for 24 hr. Growth was examined in the presence of 20 to 2000 μg/ml of the antibiotics.

R plasmid transfer. From the fresh cultures of the donor and recipient strains 0.1 ml each was given separately to 4.5 ml broth. Incubation at 37°C for 4 hr followed. After streaking the controls on plates containing the individual antibiotics, donor and recipient strains (ratio 1 : 2) were inoculated into 4.5 ml broth. The cultures were incubated at 37°C for 18 hr. The colonies grown in the presence of the different antibiotics were counted, plated, then antibiotic resistance was tested by the disk method. Transfer frequency was related to the recipient strains. Fi specificity of the R plasmids was examined with male spicific f_2 and MS_2 phages.

Phage typing of E. coli strains. Standard *E. coli* phage series [4] and T_1–T_7 phages were used.

Elimination of antibiotic resistance was examined in the broth culture of the strains under study to which chlorpromazine (40 μg/ml) was given. The cultures were incubated at 37°C for 24 to 48 hr.

Spontaneous loss of resistance was examined by keeping the donor strains and the transconjugants at room temperature for 10 months then counting the susceptible colonies. Dilutions were prepared of which 0.1 ml yielded approximately 100 colonies. After incubation at 37°C for 18 hr, replica-plating with different antibiotics was made. The spontaneous segregant colonies were subcultured several times and then checked by the disk method.

Results

S. london strains isolated earlier (from 1972 on) in County Csongrád corresponded in all their properties to those described in the Kauffmann–White scheme. Between 1976 and 1978 several outbreaks occurred; the isolates had an unchanged antigenic structure (3,10: l, v: 1,6), but were inositol negative and some of them even failed to ferment dulcitol.

While the phenotype of the epidemic strain remained stable in its biochemical functions, the antibiogram showed considerable changes. Using 10 antibiotics (ampicillin, carbenicillin, chloramphenicol, tetracycline, kanamycin, streptomycin, neomycin, polymyxin B, colistin and nalidixic acid), the original strain isolated before the 1976 epidemic proved susceptible. A strain isolated three months after the outbreak from a child hospitalized with enteritis was resistant to 3 antibiotics: chloramphenicol, tetracycline and streptomycin; no other strain displaying a similar resistance pattern was found. At the end of the first wave of the outbreak, several strains were isolated from the medical

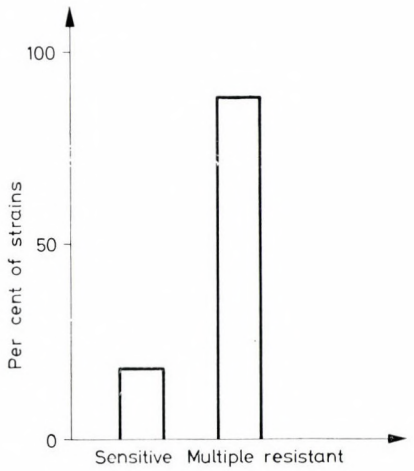

Fig. 1. Frequency of enteritis-associated sensitive and resistant *S. london* strains in hospital environment

ward and other health institutions. These strains, as compared to the original strain, developed resistance to 7 antibiotics: ampicillin, carbenicillin, chloramphenicol, streptomycin, tetracycline, kanamycin and neomycin (Table I).

A total of 87% of the multiresistant *S. london* strains were isolated from the hospital environment (Fig. 1). It is assumed that the therapeutic use of various antibiotics facilitated the selection of these strains.

The biochemical characteristics, multiple resistance and the frequent occurrence in hospital environment of these strains suggested a plasmid-mediated drug resistance. To prove this, experiments were conducted with three different strains using three methods.

1. The results of R plasmid transfer by conjugation are given in Table II. It is seen that the transfer frequency was between 1.10^{-3} and 4.10^{-4}. Transfer

Table I

Antibiogram of the epidemic S. london strains

Strain	Sm	Cm	Tc	Ap	Km	Cb	Nm	Pm	Col	Nal
S. london 5053	S	S	S	S	S	S	S	S	S	S
S. london 39082	R	R	R	S	S	S	S	S	S	S
S. london 11249	R	R	R	R	R	R	R	S	S	S

Sm = streptomycin; Cm = chloramphenicol; Tc = tetracycline; Ap = ampicillin; Km = kanamycin; Cb = carbenicillin; Nm = neomycin; Pm = polymyxin; Col = colistine; Nal = nalidixic acid; S = sensitive; R = resistant

Table II

Transfer of antibiotic resistance from S. london strains to E. coli K_{12}

Donor strain	Recipient strain	Transferred resistance	Transfer frequency
S. london 1207	*E. coli* K_{12}Nal$^-$	Ap, Cm, Sm, Tc, Km	3.7×10^{-4}
S. london 11249	*E. coli* K_{12}Nal$^-$	Ap, Cm, Sm, Tc, Km	1.5×10^{-3}
S. london 12641	*E. coli* K_{12}Nal$^-$	Ap, Cm, Sm, Tc, Km	1.3×10^{-3}

of 5 antibiotic resistances was examined (ampicillin, chloramphenicol, tetracycline, streptomycin and kanamycin). The transconjugants had become resistant to all the 5 drugs simultaneously; their phage pattern was the same as that of the recipient *E. coli* Kl2 strain. The R plasmid proved to be of fi$^-$ type.

2. The above mentioned 5 antibiotics were used to establish MIC values. MICs of the original *S. london* strain and of the *E. coli* transconjugants rendered resistant were compared. As can be seen in Table III the values were largely identical.

Table III

Minimum inhibitory concentrations (μg/ml) for the multiresistant donor and the transconjugant strains

Strains	Ap	Cm	Sm	Tc	Km
S. london 1207	2000	500	2000	500	2000
E. coli K_{12} (R 1207)	2000	500	2000	500	2000
S. london 11249	2000	500	1000	500	2000
E. coli K_{12} (R 11249)	2000	500	500	500	2000
S. london 12641	2000	500	1000	500	2000
E. coli K_{12} (R 12641)	2000	500	1000	500	2000

For abbreviations see Table II

3. Chlorpromazine [5] was used to eliminate antibiotic resistance; 100 to 200 colonies were examined in each case. About 3.5% of the examined colonies of the *S. london* strains lost their resistance to the antibiotics except tetracycline. Among the transconjugants rendered resistant, elimination was successful in 3.8% but these colonies became sensitive to tetracycline. Spontaneous loss of resistance was studied in strains kept for 10 months at room temperature. Of the donor strains 12 to 87%, of the transconjugants, 66 to 88% yielded sensitive colonies. Tetracycline resistance of the donor strains was stable in these experiments, too (Table IV).

Table IV

R plasmid elimination in the strains studied

Strains	Chlorpromazine			Spontaneous		
	Colonies examined	Colonies susceptible		Colonies examined	Colonies susceptible	
		No.	%		No.	%
S. london 1207	150	4	2.6	92	34	36.9
S. london 11249	125	6	4.8	150	18	12.0
S. london 12641	192	6	3.1	160	140	87.5
E. coli K_{12} (R 1207)	242	7	2.8	136	120	88.2
E. coli K_{12} (R 11249)	143	7	4.9	112	84	75.0
E. coli K_{12} (R 12641)	243	8	3.3	180	120	66.6

Discussion

Plasmids can be present in each cell in multiple copies. Despite their small size [6], they may constitute 20% of the total cell DNA. The cell needs extra energy to maintain this excess DNA, thus it is not surprising that it gradually loses its R plasmids in antibiotic-free medium [7]. WATANABE and LYANG [8] investigated the segregation of resistant strains in *Salmonella typhimurium*. Studying the spontaneous loss of fi^+ and fi^- plasmids, it was found that the fi^- R plasmids of the microorganism were stable, whereas the fi^+ types became segregated with high frequency and remained stable only in *E. coli* strains [9, 10]. YOSHIKAWA *et al.* [11] investigated the relationship between the host cell and R plasmids in *Salmonella*, *Shigella* and *E. coli* strains. They found that the R plasmids isolated from salmonellae were mostly of the fi^- type.

Our experiments seem to have proved that the DNA genes responsible for the multiple resistance of *S. london* strains isolated during the epidemic were located on the plasmid. Resistance transfer to the transconjugants of the 5 antibiotics examined occurred invariably simultaneously as did also the loss of resistance. The stability of tetracycline resistance, in agreement with the observation of other authors [10], might be due to the more intensive attachment of the tetracycline locus to the host chromosome or to its location on a separate plasmid.

Several papers have dealt with the multiple resistance in human and animal *Salmonella* strains. The use of antibiotics as feed additives and for therapeutic purposes in veterinary practice exert a selection on the multiresistant strains carried by the animals. Since intergeneric plasmid transfer is common, transfer to strains of human pathogenicity represents a serious dan-

ger. DUCK *et al.* [12] analysed the resistance of different *S. typhi-murium* sero and phage-variants isolated in Canada in 1975 and 1976. They found that 61.7% of the equine, bovine and avian strains carried resistance to multiple antibiotics. NEU *et al.* [13] examined the antibiograms and serotypes of human and animal *Salmonella* strains isolated in the north-eastern states of the USA. They, too, found that multiple resistance was most frequent in *S. typhi-murium*, in 58% of the human and 80% of the animal strains. In 1968 and 1969 only 12.5% of the strains carried tetracycline resistance. In general, the resistance to 6 drugs (ampicillin, chloramphenicol, streptomycin, tetracycline, kanamycin and sulphonamide) was transferable. TIMONEY [14] reported that only 12% of the isolates from diseased animals (cow, calf, horse, dog, cat) were susceptible. Of the resistant strains, 91% carried multiple resistance but one-third of the resistances was transferable only at 28 °C. ANDERSON [15] prepared a map of R plasmids related to the territorial distribution of *Salmonella* strains, and found that specific R plasmid groups were dominating at different parts of the world. As *S. typhi-murium* strains circulate, they may acquire new R plasmids in human or animal hosts through recombination with other enteric bacteria. RISCHE *et al.* [16] studied the manner of the spread of infection from animal to man and vice versa; they also discussed the possibilities of R plasmid selection in hospital, urban and rural environments, animal husbandry, plant production, among workers of the antibiotics industry and animal feed production.

The predominance of *Salmonella* serotypes varies from year to year all over the world. Nevertheless, *S. typhi-murium* is usually listed among the most common ones isolated from foodstuffs of animal origin, whereas *S. london* strains were not mentioned in this group until 1975. TAKÁCS [17] reported on *Salmonella* strains isolated in Hungary in 1974 and 1975; in 1974 *S. london* was not in the group of the most frequent *Salmonella* isolates but in 1975 it already took the fifth place. MILCH *et al.* [18] investigated the drug sensitivity of 2400 *S. typhi-murium* strains isolated in Hungary; 4.5% were multiresistant and of these the resistance was plasmid-mediated in 80.5%.

In the outbreaks described in this paper, epidemiological evidence also supported the role of raw meat and meat products in the spread of the infection. The strains derived from foodstuffs were, however, all drug sensitive except a single one isolated in the course of environmental screening. It was first in a hospital environment that a multiresistant strain was revealed; this was then followed by several others. A total of 83% of the hospital strains carried multiple resistance. Unfortunately, after the appearance of multiresistant strains, there was no possibility to examine the intestinal flora of the patients for the presence of bacteria with similar multiple resistance. Therefore, plasmid-mediated resistance of the epidemic strain could only be examined *in vitro*.

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LACK OF CORRELATION BETWEEN INTERCALATION AND PLASMID CURING ABILITY OF SOME TRICYCLIC COMPOUNDS

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Some tricyclic psychotropic drugs are known to have plasmid curing activity. The interaction with DNA of three plasmid curing (chlorpromazine, amitriptyline, imipramine) and four ineffective (methylene blue, 7,8-dioxo-chlorpromazine, thiazinamium, chlorpromazine sulphoxide) compounds was studied by fluorescence polarization and circular dichroism methods. Among the seven compounds three, namely chlorpromazine, 7,8-O_2-chlorpormazine, and methylene blue showed an intercalation effect. Other phenothiazines such as chlorpromazine sulphoxide and thiazinamium were not able to intercalate into *Escherichia coli* DNA, neither did the plasmid curing drugs amitriptyline and imipramine. It is concluded that the plasmid curing ability is not necessarily related to the intercalation ability.

Several investigators have studied the interaction of DNA with small molecules and some possibilities of specific interaction are known. Recently we have been interested in the effect of various phenothiazine derivatives on certain aspects of DNA replication in bacteria. Phenothiazines have a multifocal action on biological systems and many of these compounds are widely used in psychiatric disorders. Moreover, phenothiazine derivatives exert some antibacterial effect in several species [1–3]. Detailed genetic studies have shown that R-factor and F'lac factors in *Escherichia coli* can efficiently be cured by chlorporomazine [2–4]. Besides, we have selected lon-mutants of *E. coli* with a rather high frequency in the presence of chlorpromazine, promethazine [5] and imipramine [6]. These data indicate that the tricyclic drugs may interact with DNA *in vivo*.

The bacterial plasmids are responsible for the polyresistance of bacteria to antibiotics and the elimination of this resistance is an important question in chemotherapy. One possible method of plasmid elimination is the inhibition of plasmid replication by tricyclic compounds. According to recent investigations the inhibitory effect is probably based on intercalation into the plasmid DNA (e. g. acridine orange [7–10]). The aim of the present study was to test the validity of this supposition. Therefore, we investigated the binding to DNA of three plasmid curing compounds: chlorpromazine [2, 4]; imipramine [6, 11]; and amitriptyline [11], and of the four ineffective compounds methylene blue [8, 11]; chlorpromazine sulphoxide [11, 12], 7,8-dioxo-chlorpromazine [13] and thiazinamium [14] by fluorescence polarization and CD techniques.

Materials and methods

E. coli MRE 600 DNA was kindly provided by Dr. A. UDVARDY (Institute of Biochemistry, Biological Research Centre, Szeged); poly-A potassium salt was a Sigma (USA) product; 7,8-dioxo-chlorpromazine HCl was a gift from Dr. A. A. MANIAN (Psychopharmacology Research Branch, Department of Mental Health, Rockville, Md. USA). Chlorpromazine sulphoxide was prepared according to FISCHMAN and GOLDENBERG [15]. Chlorpromazine HCl (Hibernal®), imipramine HCl (Melipramine®), amitriptyline HCl (Teperin®) were obtained from EGYT Pharmaceutical Works (Budapest, Hungary), methylene blue from Reanal (Budapest, Hungary) and thiazinamium methylsulphate (Multergan®) from Specia (Paris, France). Their chemical structure is shown in Fig. 1. In the spectroscopic studies the following concentrations were used: 1.6×10^{-4} M DNA-P and poly-A-P (where DNA-P and poly-A-P refer to gram-atoms of phosphorus per liter), while the compound concentration ranged from 1.0×10^{-5} to 5.0×10^{-5} M. The ratio r, defined as

$$r = \frac{\text{(compound concentration)}}{\text{DNA-P or poly-A-P (concentration)}}$$

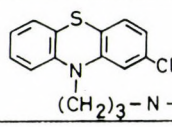
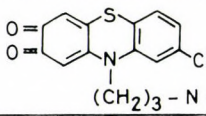
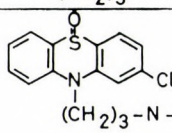
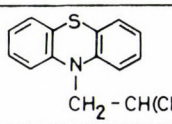
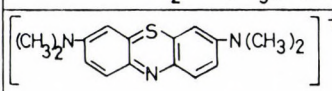
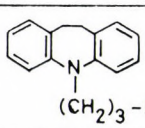
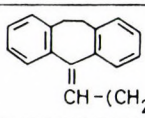
Compounds	
Formula	Name
	Chlorpromazine
	7.8 - Dioxo-chloropromazine
	Chlorpromazine sulphoxide
	Thiazinamium
	Methylene blue
	Imipramine
	Amitriptyline

Fig. 1. Chemical structure of the compounds tested

corresponded to 0.06, 0.13 and 0.31, respectively in this concentration region. With these concentrations the extinction of samples was $E < 2$ in a $d = 1$ cm cuvette. All the chemicals were dissolved in a buffer solution containing 5×10^{-3} M NaCl, 5×10^{-3} M Tris and 5×10^{-4} of EDTA. The DNA ($\sim$1 mg/ml) was prepared by the method of Chambon (D) [1]. The pH of the solutions was 7.0 ± 0.2.

Fluorescence polarization. Fluorescence polarization measurements were carried out on a Perkin–Elmer MPF-3 spectrofluorimeter. The excitation and fluorescence maxima given in Table I were selected for excitation and observation. If two fluorescence maxima were present, the measurements were performed at both wave-lengths. The degree of fluorescence polarization (P) is given by the expression

$$P = \frac{F_{II} - F_I}{F_{II} + F_I}$$

where F_{II} and F_I denote the observed fluorescence intensities in the parallel and perpendicular directions, respectively. The data were corrected for the inherent polarization of the instrument.

Circular dichroism. For circular dichroism measurements a Jasco-40 C automatic recording spectropolarimeter was used. Molar ellipticities, defined as

$$\Theta = \frac{\Theta'}{c \times d} \times 100,$$

are expressed in $\deg \times cm^2 \times decimole^{-1}$ (c, d and Θ' denote the concentration of DNA, the pathlength and the observed ellipticity). Measurements were performed at room temperature.

Table I

Absorption and fluorescence maxima of the DNA-compound complexes in water at pH 7.0±0.2

Compounds*	Absorbance max (nm)	Fluorescence max (nm)**
DNA	258	360
DNA + chlorpromazine	258	380; 455
DNA + chlorpromazine sulphoxide	242; 267	380
DNA + 7,8-dioxochlorpromazine	264; 520	380
DNA + thiazinamium	253	375; 445
DNA + imipramine	256	370
DNA + amitriptyline	249	375
DNA + methylene blue	~253; 270	
	607; 668	375; 695

* Solutions were 2.5×10^{-5} M for the compounds and 1.6×10^{-4} M for DNA

** No correction was made

Results

As the absorption of the compounds is roughly in the range of the absorption of DNA, absorption and fluorescence measurements do not give conclusive information about the intercalation ability of these materials. A more straightforward approach is provided by fluorescence polarization (P) and circular

dichroism (CD). If a compound intercalates into the DNA, the following spectral changes can be observed:

I. (a) A significant increase in the fluorescence polarization degree as compared to the poly-A bound one.

(b) A considerable decrease in the fluorescence polarization degree above a certain concentration of the compounds.

II. Changes in the CD spectrum.

The difference in the fluorescence polarization degree when the compounds are bound to DNA and poly-A, respectively,

$$\Delta P = P_{DNA+compound} - P_{poly\text{-}A+compound}$$

is shown at different r values in Fig. 2. If $r > 0.2$, the intercalation sites may be saturated [17] and, thus the possibility of external binding can significantly be increased, resulting in a decrease of the fluorescence polarization degree. The curves in Fig. 2 could be divided into two groups. The first group (curves 1–4) gave a pronounced decrease with the increase in concentration. For each compound of group I the difference in ΔP between the highest and lowest concentration exceeded 0.1 in every independent repetition of the measurements. The behaviour of these compounds is obviously different from that of compounds in the second group (curves 5–7) since the same parameter was always smaller than 0.04. The experimental error in determining ΔP was ± 0.02, therefore we tend to conclude that changes in second group were not significant. Changes in first group were, however, beyond the experimental error. For the ΔP values of the complexes which have another fluorescence maximum at higher wave-length (see Table I), a similar concentration dependence was found, although the effect was smaller.

All the compounds absorb in the same region as DNA, while 7,8-dioxochlorpromazine and methylene blue also absorb at 520 nm (broad and weak band) and at 666 and 617 nm, respectively; neither the UV nor the visible bands are, however, optically active.

E. coli DNA has a positive and a negative CD band at 275 nm and 245 nm, respectively. If the compound interacted with DNA the resulting CD exhibited similar changes in intensity at both bands. No significant wavelength shift (< 5 nm) was observed as compared to the CD of DNA. Methylene blue when complexing with DNA showed an additional induced optical activity at 585 nm (positive) and 670 nm (negative). For an easier comparison of the effect of the compounds, the molar ellipticity values measured at 275 nm are given in Fig. 3. On the basis of the CD results there is some evidence of the observed change being due to intercalation of compounds; in the case of chlorpromazine 7,8-dioxo-chlorpromazine and methylene blue, while other drugs such as amitriptyline, imipramine, chlorpromazine sulphoxide and thiazinamium failed to intercalate.

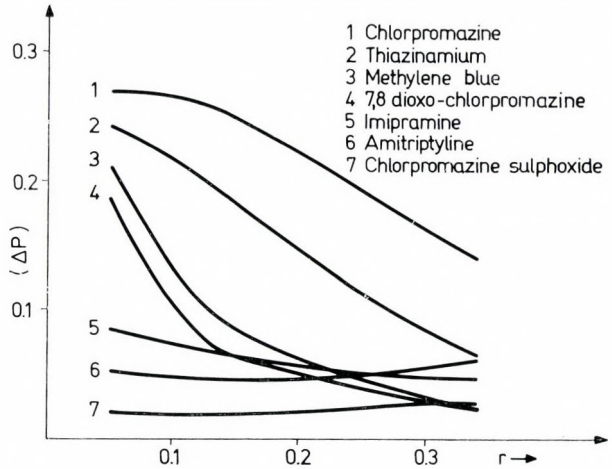


Fig. 2. Fluorescence polarization degree differences (ΔP : DNA + compound with respect to poly-A + compound) at different compound/DNA rations (r)

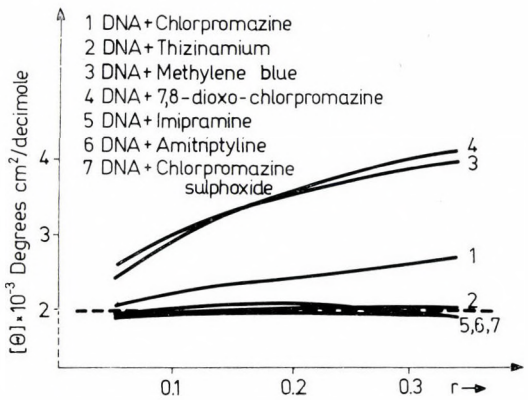


Fig. 3. Molar ellipiticities (Θ) of DNA + compound at different compound/DNA ratios (r) measured at 275 nm (full lines). For the sake of comparison the CD intensity of DNA (r = 0) is shown by dashed line

Discussion

DNA-chlorpromazine complexes have been studied by a variety of techniques. Such studies are of importance for a number of reasons. A principle biological interest centres around the attempt to understand the antibacterial, plasmid curing and possible mutagenic activity of chlorpromazine on bacteria. It has been suggested that chlorpromazine might intercalate into bacterial DNA because its chemical and structural similarity to acridine dyes.

Nevertheless, it was of interest that some chlorpromazine metabolites (chlorpromazine sulphoxide and 7,8-dioxo-chlorpromazine) are not able to

eliminate the F'lac plasmid of *E. coli* [11–13]. On the other hand some antidepressants (e.g. amitriptyline and imipramine) which have no planar structure, exert a good plasmid curing activity [11, 14].

Earlier studies on chlorpromazine [18–22] and methylene blue [18, 20, 23] reported on the intercalation of these compounds. The combination of fluorescence polarization degree and CD data suggests that 7,8-dioxo-chlorpromazine most probably intercalates into DNA. In the case of amitriptyline,

Table II

Comparison of intercalation and plasmid curing abilities of some tricyclic compounds

Compounds	Plasmid curing	Intercalation
Chlorpromazine	+	+
Chlorpromazine sulphoxide	−	−
7,8-Dioxochlorpromazine	−	+
Thiazinamium	−	−
Imipramine	+	−
Amitriptyline	+	−
Methylene blue	−	+

imipramine and chlorpromazine sulphoxide the changes are too small, suggesting an external binding of the compounds to DNA. Thiazinamium shows a different behaviour: fluorescence polarization degree differences (ΔP) versus r reveal intercalation; no change in the CD spectrum was, however, observed. This may be interpreted by supposing a special and strong external binding of thiazinamium to DNA.

In Table II the intercalation of each compound is shown with its F'lac$^-$ plasmid curing ability on *E.coli*. On the basis of these results it is supposed that the plasmid curing effect of the tested psychoactive drugs differs from that of acridine. We suggest that the molecular basis of plasmid elimination by psychotropic drugs may be due to their intercalation directly into the bacterial membrane on which the plasmid replication occurs and the altered membrane structures do not allow for plasmid replication.

It is known that some positively charged local anaesthetic molecules e.g. procaine, furthermore chlorpromazine and morphine which also have a local anaesthetic effect, strongly bind to the cell membrane [24–28].

There is some other evidence against the exclusive role of the intercalation into DNA: methylene blue antagonizes the plasmid elimination effect of acridine [8] while it increases the plasmid curing activity of imipramine [6]. The lack of correlation allows the conclusion that plasmid curing cannot be simply explained by a direct interaction between DNA and the compound.

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ENTEROTOXIGENICITY OF AEROMONAS STRAINS IN SUCKLING MICE*

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The enterotoxigenicity of 170 *Aeromonas* strains isolated from different sources (food poisoning, random food sampling, water, faeces) was examined by the suckling mouse test. The strains were grown on Syncaye culture medium covered with sterilized membrane for Kiil-kidney. The culture supernatants were inoculated orally. Ileal loop dilatation was compared to that produced by the international standard enterotoxic *Escherichia coli* B7A (O148 : H28) and B2C (O6 : H16) strains. Of the 87 *Aeromonas hydrophila* strains 69, of the 76 *Aeromonas punctata* subsp. *caviae* strains 9, the 6 *Aeromonas punctata* subsp. *punctata* strains 5, and 1 *Aeromonas salmonicida* subsp. *achromogenes* gave a positive reaction in the test.

Since the 60s cases were observed where aeromonads have been made responsible for mild or serious enteric diseases in adults and children [1–8]. Some of the illnesses resembled cholera. Contaminated drinking water or bathing water was often found to be the source of infection.

It has been shown earlier that aeromonads can be transmitted by foodstuffs, too. In food poisoning cases mainly soups, further potato, rice or noodles boiled in water contained aeromonads often in almost pure culture, in an order of magnitude of 10^7 to 10^9/g. No other enteric pathogens could be detected in the food or stool specimens [9].

DE and CHATTERJEE's rabbit ileal loop model [10] has been used by several investigators to test the enterotoxigenicity of aeromonads isolated from patients with enteritis [11–13]. Some of the strains produced a loop dilatation similar as did the enterotoxin of *Vibrio cholerae*.

In our laboratory the suckling mouse (SM) model described by DEAN *et al.* [14] is regularly applied for testing enterotoxigenicity of *Escherichia coli* strains. In the experiments reported in this paper the same method was used for assaying aeromonads isolated from cases of food poisoning and other sources.

Materials and methods

Strains. The international standard. *E. coli* B7A (O148 : H28), B2C (O6 : H16), 1624 (O144 : K? : H–), 4608 (O143 : K? : H–) strains were a gift from H. L. DUPONT (University of Maryland, School of Medicine, Baltimore, Maryland, USA). *Aeromonas* strains derived from food samples causing intoxication, random foodstuff sampling, driking water, surface water, sewage and faeces.

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Culture medium. Syncaye: Synthetic Broth (AOAC, Difco) 1.7%, Casamino acid (Difco) 1%, Yeast extract (Difco) 0.3%, dextrose (Reanal) 0.1%, 1 ml salt solution ($FeCl_3$, 0.5 g; $MnSO_4$, 3.6 g; NH_4Cl, 11.4 g; $ZnSO_4$, 0.22 g; $CuSO_4 \cdot 5H_2O$, 50 mg dissolved in 10 ml distilled water) added to 1000 ml culture medium. Noble agar (Difco) 1%, pH 7.5 $\pm$ 0.1; sterilization at 115 °C for 20 min.

Cultivation. The Syncaye plate medium (in Petri dish 10 cm in diameter) was covered with sterilized membrane for Kiil-kidney [15]. Two and half ml of the bacterial suspension washed with physiological saline off a 18 hr slant culture was inoculated. The plates were incubated at 32 °C for 24 hr. The cells in 2 ml total volume, if necessary supplemented with physiological saline, were picked from the membrane and centrifuged at 15 000 *g* at 4 °C for 30 min. The pure supernatants were stored at —25 °C until usage.

Suckling mouse test. Three to 4 days old suckling Balb/C mice were given through a gastric tube, millilitres of the supernatants corresponding to 1/20 of their bodyweight. Adjustment of the dose to bodyweight served to diminish errors due to weight differences. The animals were kept at room temperature for 3 hr then killed by chloroform. The intestinal tract was removed and the ratio of gut weight to bodyweight was calculated. Initially, 4 mice were used for each supernatant, later only 2 because the results were usually in good agreement. If there was a discrepancy exceeding 15% between the results with the 2 animals, the tests were repeated. Gut weight to bodyweight ratios above 0.085 were considered positive, those below that value, negative.

Results

Before treating the data in detail, the mouse gut reaction is illustrated in Fig. 1.

First, *Aeromonas* strains isolated from food samples causing intoxication were examined. Distribution of the 23 strains in relation to the reaction in the SM test and the species under study is shown in Table I.

Table I

Aeromonas strains causing food poisoning. Reaction in the SM test

Aeromonas species (Bergey 1974)	SM test		Total
	positive	negative	
A. hydrophila subsp. *hydrophila*	9	1	10
A. punctata subsp. *punctata*	1	1	2
A. punctata subsp. *caviae*	1	10	11
Total	11	12	23

Enterotoxigenic strains were present in the incriminated food samples in every case of food poisoning but strains giving a negative reaction were also found. The majority of *A. hydrophila* subsp. *hydrophila* strains proved enterotoxic, while most of the *A. punctata* subsp. *caviae* strains gave a negative response.

To clarify this relationship, further 147 *Aeromonas* strains isolated from foodstuffs, drinking water, sewage, or faeces were assayed by the SM test.

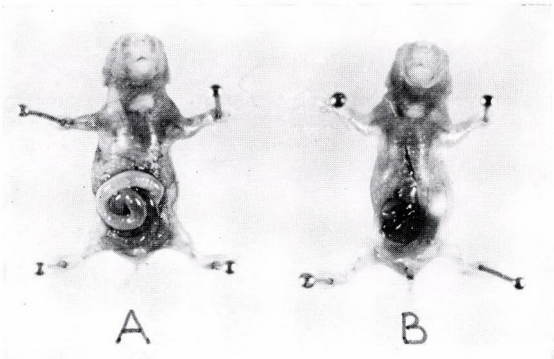

Fig. 1. Mouse intestinal reaction. A = positive; B = negative. (Evans blue given together with the supernatant strains the gut darker)

Table II shows the values of the SM test obtained with the international standard *E. coli* strain. The results agreed with those reported in the literature. The non-enterotoxigenic control *E. coli* strains causing a shigellosis-like disease that were negative in the rabbit-loop test [16], gave negative results in mice.

The results of the SM tests with the 170 *Aeromonas* strains are demonstrated in detail in Table III. The 0.085 limit value of the SM test was determined on the basis of the data of the 67 *A. punctata* subsp. *caviae* strains negative in the SM test. The mean value was increased by the threefold deviation in order to obtain a reliable positive gut weight to bodyweight ratio. This practically did not differ from the 0.083 value accepted for *E. coli* strains, above which the results are considered positive [17, 18].

In our series, 79% of the 87 *A. hydrophila* and 12% of the 76 *A. punctata* subsp. *caviae* strains proved enterotoxigenic in the mouse. Only 6 *A. punctata*

Table II

SM test controls

Controls		SM test*
Positive	*E. coli* B7A (0148 : H28)	0.113±0.015
	E. coli B2C (O6 : H16)	0.108±0.014
Negative	*E. coli* 1624 (O144 : K? : H-)	0.055±0.003
	E. coli 4608 (0143 : K? : H-)	0.058±0.005
	Physiological saline	0.058±0.006

* 4 parallels each

Table III

Results of SM tests with aeromonads

Aeromonas species (Bergey 1974)	No. of strains	SM positive	$\bar{x}$	SM negative	$\bar{x}$
A. hydrophila subsp. *hydrophila*	61	46	0.105±0.011	15	0.069±0.010
A. hydrophila subsp. *anaerogenes*	26	23	0.104±0.012	3	0.065±0.009
A. punctata subsp. *caviae*	76	9	0.101±0.009	67	0.061±0.008
A. punctata subsp. *punctata*	6	5	0.108—0.010	1	0.053
A. salmonicida subsp. *achromogenes*	1	1	0.105		
Total	170	84		86	

subsp. *punctata* occurred in the material, nevertheless 5 of these were enterotoxigenic as was the one *A. salmonicida* subsp. *achromogenes* strain tested.

While carrying out the experiments, two highly toxic strains were found. The mice inoculated with the supernatants of the two *A. hydrophila* subsp. *hydrophila* strains, one isolated from a stool sample the other from a food sample, died in about 30 min. Necropsy revealed gross haemorrhagic gut necrosis, extensive intestinal and peritoneal fluid accumulation. The intestinal tract could not be removed because it disintegrated on manipulation. The SM test was repeated with two-scale dilutions of the supernatants. The results are shown in Table IV.

Table IV

Titration of the supernatants of Aeromonas strains 30 and 119 by SM test

Strain number	SM values at supernatant dilutions					
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64
30	0.122	0.119	0.102	0.077	0.064	0.066
119	0.102	0.100	0.082	0.070	0.065	0.057

Supernatants of 1 : 2 dilutions no longer killed the mice but intestinal haemorrhages could still be observed; 1 : 4 dilutions did not produce haemorrhage. The SM tests with the supernatants of strain 119 elicited gut dilatation when used up to a dilution of 1 : 4, of strain 30 up to a dilution of 1 : 8.

Distribution according to origin of the strains is given in Table V. The data show that among the strains isolated from drinking water, enterotoxigenic species had a higher frequency.

Table V

Distribution of Aeromonas strains according to origin and species

Aeromonas species (Bergey 1974)	Foodstuffs	Drinking water	Surface water	Sewage, faeces	Total
A. hydrophila subsp. *hydrophila*	20	34	4	2	60
A. hydrophila subsp. *anaerogenes*	13	5	1	1	20
A. punctata subsp. *punctata*	3	7	—	—	10
A. punctata subsp. *caviae*	65	1	3	10	79
A. salmonicida subsp. *achromogenes*	1	—	—	—	1
Total	102	47	8	13	170

Discussion

Using our method suitable for serial examinations, the enterotoxic effect of aeromonads isolated from foodstuff, water and stool specimens was investigated by the SM test. Of the 170 strains under study, 50% produced the same loop dilatation as did the international standard enterotoxic *E. coli* strains. Out of the 84 positive strains 82% were *A. hydrophila*, of the 86 negative ones 78% *A. punctata* subsp. *caviae*. Enterotoxicity could be demonstrated in 74% of the 87 *A. hydrophila* strains. ANNAPURA and SANYAL [11] obtained similar results using the rabbit ileal loop assay. They found that 82% of their 57 human *A. hydrophila* strains were enterotoxigenic. It seems that the rabbit gut and the mouse gut model are of the same value for demonstrating enterotoxicity of aeromonads, depending naturally on the culture media and the testing procedures applied.

BOULANGER *et al.* [19] also used the SM test for the examination of aeromonads isolated from healthy and diseased fish: 19 of the 21 strains gave positive reactions. As to the bacterial species, no comparison can be made, because they used the identification method of POPOFF and VÉRON [20]. Enterotoxigenicity of *Aeromonas* strains cultivated from healthy fish is of special interest in view of the report of KALINA [21] who described *Aeromonas*-related toxicoinfection subsequent to the consumption of fish.

It has been shown that aeromonads produce in addition to enterotoxic substance(s) other extracellular toxic substances (necrotoxin, haemolysin). WADSTRÖM *et al.* [13] reported that the effect of the latter can be eliminated by heat treatment at 56 °C for 10 min. In this way enterotoxicity can be differentiated since enterotoxin becomes inactivated only at 80 °C for 10 min. In con-

trast, our studies demonstrated that enterotoxic activity measurable in the SM test was eliminated at 56 °C for 10 min.

Our preliminary experiments on the presence of other toxic substances that may influence the result of the SM test revealed that supernatants with an enterotoxic effect did not always haemolyse sheep erythrocytes or produced a necrotoxic effect in the skin test of infantile mice. With the two highly toxic strains, the appearance of the necrotoxic and of the enterotoxic activity could be distinguished by serial dilutions of the supernatants. DONTA and HADDOW [22] also observed haemorrhagic necrosis from some *A. hydrophila* filtrates in rabbit ileal loop studies, though none of the filtrates dilated the intestines. They found that most *A. hydrophila* strains were cytotoxic in tissue culture. The cytotoxic substance became inactivated at 50 °C for 10 min, as did the enterotoxigenic substance demonstrated by us in the mouse model. CRAIG [23] observed that cholera enterotoxin is just as sensitive to heat treatment. Clarification of the problems requires a great number of investigations.

The present results and other data [11–13] concerning the enterotoxigenic effect of aeromonads support the importance of *A. hydrophila* strains in human enteric disease. The findings indicate that enterotoxigenic substances may have a role in infections caused by aeromonads (a genus related to vibrios), as is the case in enterotoxic enteropathies due to *V. cholerae*, *V. parahaemolyticus*, *E. coli*, *Clostridium perfringens* and *Shigella* [5].

Our results also suggest that enterotoxigenic aeromonads are mainly transmitted by water and via the water also by food and may thus cause acute enteric disease.

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ASSOCIATION OF HUMAN ENTERIC PATHOGENICITY AND MOUSE LUNG TOXICITY OF ESCHERICHIA COLI*

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Mouse lung toxicity of 439 strains (431 *Escherichia coli*, 1 *Shigella dysenteriae* 1, 1 *Enterobacter cloacae*, 5 *Vibrio* sp., 1 *Klebsiella)* was compared to other pathogenicity tests (mouse virulence, enterotoxicity, guinea pig eye test), to serogroup distribution, loss of virulence on storage, origin and haemolytic activity. Mouse lethality was highest in serogroup O4 ($p < 0.001$), O18a,c ($p < 0.001$); serogroups O6, O20, O75, O115, O147 were next in order. *E. coli* serogroups O19, O26, O28a,b, O32, O51, O53, O55, O73, O78, O79, O83, O105, O111, O112, O114, O117, O119, O124, O129, O136, O142, O148 failed to show lung toxicity. Strains O4 and O18 isolated at different periods of time did not differ significantly in the lung test ($p = 0.05$, $p = 0.01$, $p > 0.1$, $p = 0.05$, $p > 0.1$). There was no significant difference between strains isolated from the stools of patients with enteritis and of healthy individuals ($p = 0.1$, $p > 0.99$) and between those isolated from all faecal specimens and from extraintestinal samples ($p = 0.05$, $p > 0.3$). There was no correlation between lung toxicity and other pathogenicity tests. Since strains isolated from healthy individuals were also toxic for mice, a positive lung test cannot be considered a criterion of the aetiological role of the agent.

In the last two decades, besides serotyping [1–3] the importance to prove the pathogenicity of *Escherichia coli* has been in the foreground. Mouse virulence of strains causing extraintestinal infections [4–6], epithelial penetration [7–9] and enterotoxicity [10–17] of the enteropathogenic strains, immunochemical properties of the strains [18] and antibody titres of patients [19, 20] have been investigated. In spite of this attempts to prove the pathogenicity of different strains isolated during outbreaks have failed.

In view of the occasional presence of nosocomial strains it is of major importance to prove their pathogenic role in the outbreak. Permanent presence of *E. coli* in the intestinal flora made it necessary to elaborate suitable new methods to ascertain the aetiological role of the isolates in enteric diseases such as enteritis necrotisans, enterocolitis, etc.

VOINO-YASENETSKY and KHAVKIN [21] have shown by means of the "lung test" that *E. coli* strains causing dysentery-like diseases and inoculated intranasally penetrate into the bronchial and bronchiolar epithelium of young mice. AVDEEVA *et al.* [22] found that certain *E. coli* strains isolated from newborns with acute diarrhoeal disease belonging to none of the classical enteropathogenic groups caused progressive haemorrhagic lung oedema in mice fol-

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lowing intranasal infection. Properties of the toxic material produced by these *E. coli* strains was studied by KÉTYI *et al.* [23].

Our investigations were aimed at demonstrating a correlation between mouse toxicity and human pathogenicity.

Materials and methods

The strains tested were isolated in our laboratory between 1974 and 1977 from faecal samples of infants and adults who were either healthy (57) or suffering from enteritis (296). Further sources were: wound swab (27), cerebrospinal fluid (11), urine (6), throat swab (26), other pathological materials from patients with extraintestinal diseases (8). The diseases occurred either in hospitalized patients or among the population. Most of the isolates were *E. coli* (431); other bacteria were represented by *E. cloacae* (1), *Vibrio* sp. (5), *S. dysenteriae* 1 (1) and *Klebsiella* (1). Further strains tested were: enterotoxin-producing *E. coli* reference strains P99, P105, B7a (isolated by SMITH and DUPONT, received from S. STAVRIC, Health Welfare Canada, Ottawa); guinea pig-eye-positive *E. coli* strains 6600, 6730 ("serotype 792" isolated by TRIFONOVA from patients with dysentery-like disease, received from H. MILCH, National Institute of Hygiene, Budapest); *E. coli* strains (3 and 4) from infantile enteritis and *E. coli* strain Kreka showing lung toxicity (I. KÉTYI, Institute of Microbiology, University Medical School, Pécs).

Broth cultures. Stock cultures were inoculated onto blood agar plates. After incubation at 37 °C for 18 hr, three colonies were picked up, inoculated into broth and incubated at 37 °C for 18 hr.

Haemolysin producing capacity was tested on blood agar plates containing 5% defibrinated ox blood.

Serotyping was performed according to the method described earlier [24].

Lung test. CFLP albino mice weighing 12–14 g, under superficial ether anaesthesia were infected with 0.05 ml of a broth culture intranasally. Each group contained 5 mice.

Enterotoxin test was performed according to the method described earlier [25].

Guinea pig eye test was carried out by SERÉNY's method [26].

Statistical analysis. The data were analysed using the 2×2 contingency tables and examined for significance by the χ^2 test [27].

Results

Lung toxicity of 439 strains was examined in 2434 mice. First of all, the lung toxicity of 40 strains was tested (Table I). The strains were: *E. coli* belonging to known serogroups, *E. cloacae*, *S. dysenteriae* 1, *Vibrio* sp., *Klebsiella*, received from various sources. Most of the isolates (28 out of 40) were enterotoxigenic; 4 of them gave a positive guinea pig eye test and 15 among them were haemolytic.

Correlation between the above-described properties of the strains and mouse toxicity was studied. Results are shown in Table I. The following strains isolated either from patients or healthy individuals uniformly caused fatal haemorrhagic pneumonia within 24 hr in mice: *E. coli* serogroups O4, O18a,c, (Lsz 14c, 17968, 47683, 59426, Kreka, 27245, 27254, 27566/2, 1536), *Vibrio* sp. with haemolytic activity (81938, 69521, 53371, 38246, 72473) and non-haemolytic but guinea pig eye positive *E. coli* not determined serogroup, O28 and O143 (887, 6600, 6730, 85170). Some enterotoxigenic strains showed also lung toxicity (M, 40116).

Table I

Comparative study of enterotoxicity, haemolytic activity, guinea pig eye pathogenicity and lung toxicity of strains

Strain	Source	Species (serogroup)	Entero-toxicity	Guinea pig eye test	Haemo-lysis	Lung test* died/ infected mice
Lsz 14c	F, E	*E. coli* O4	+	—	+	6/10
67643/3	F, E	*E. coli* O75	+	—	—	0/10
74971	F, E	*E. coli* O119	+	—	—	0/10
23473	C, M	*E. coli* O78	—	—	—	0/10
M56899	F, E	*E. coli* O114	+	—	—	0/10
8013	F, E	*E. coli* O148	+	—	—	1/10
62268	F, E	*E. coli* O111	+	—	—	0/10
17968	C, M	*E. coli* O18a,c	+	—	—	1/10
27272	C, M	*E. coli* O83	+	—	—	0/10
40116	F, E	*E. coli* O111	+	—	—	2/10
Lsz 463b/2	F, E	*E. coli* O78	+	—	—	0/10
Lsz 233a	F, E	*E. coli* O117	+	—	—	0/9
Lsz 326e	F, E	*E. coli* O75	+	—	—	0/9
47683	F, E	*E. coli* O18a,c	+	—	+	8/9
59426	F, E	*E. coli* O18a,c	+	—	+	10/10
85170	F, E	*E. coli* O143	+	+	—	6/10
887	F, E	*E. coli* ND	+	+	—	2/10
887/v	F, E	*E. coli* ND	—	—	—	2/10
27245	F, E	*E. coli* O4 : K12	—	—	+	4/10
27254	F, E	*E. coli* O4 : K3	—	—	+	10/10
27566/1	F, E	*E. coli* O142	—	—	—	3/10
27566/2	F, E	*E. coli* O4 : K12	—	—	+	8/10
1536	F, H	*E. coli* O4 : K12	—	—	+	8/10
56547	F, E	*E. coli* O26	·	—	—	0/10
3	KÉTYI, I.	*E. coli* O26	+	—	—	0/10
4	KÉTYI, I.	*E. coli* O26	+	—	—	0/10
Kreka	KÉTYI, I.	*E. coli* O4	·	—	+	13/15
6600 ("serotype 792")	TRIFONOVA, A.	*E. coli* O28	+	+	—	8/10
6730 ("serotype 792")	TRIFONOVA, A.	*E. coli* O28	+	+	—	7/9
P99	SMITH, H. W.	*E. coli* O141	+	—	+	3/10
P105	SMITH, H. W	*E. coli* O138	+	—	+	5/10
B7a	DUPONT, H. L.	*E. coli* O148	+	—	—	1/10
M	F, E	*S. dysenteriae* 1	+	—	—	3/10
27247	F, E	*Klebsiella*	—	—	—	1/10
39017	F, E	*E. cloacae*	+	—	—	0/10
81938	F, E	*Vibrio* sp.	+	—	+	4/9
69521	F, E	*Vibrio* sp.	+	—	+	5/9
53371	F, H	*Vibrio* sp.	—	—	+	4/10
38246	F, H	*Vibrio* sp.	+	—	+	8/10
72473	W	*Vibrio* sp.	—	—	+	8/10

F = Faeces; C = Cerebrospinal fluid; E = Enteritis; M = Meningitis; H = Healthy individual; W = Surface water;
* 24-hour results of two experiments
· not tested
ND O antigen not determined

In the second part of the experiments we studied the number of mice dying within the first four hours after inoculation with 399 strains from clinically well-defined material representing 32 serogroups. Results are shown in Table II.

Whether a loss of virulence occurred on storage, the data related to *E. coli* O4 and O18a,c were analysed, as in these two serogropus was the incidence of mouse lethality the highest.

Mouse toxicity of strains isolated earlier compared to that of strains isolated in 1977 did not differ essentially either in serogroup O4 (1974, $p=0.05$; 1975, $p = 0.01$) or in serogroup O18a,c (1974, $p > 0.1$; 1975, $p = 0.05$; 1976, $p = 0.1$).

Subsequently, the pathogenicity of strains from enteritis patients and from healthy individuals was examined on the basis of the data of *E. coli* O4 and O18a,c serogroups. The results were $p = 0.1$ for serogroup O4 and $p > 0.99$ for O18a,c.

Lung toxicity of strains found in stool samples and of those isolated from extraintestinal diseases was compared for the same serogroups. The results were $p = 0.05$ and $p > 0.3$, respectively.

As to the mouse lethality of haemolytic and non-haemolytic strains, data of all the *E. coli* strains tested showed that haemolytic strains were significantly more frequently lethal than non-haemolytic ones ($p < 0.001$).

Comparison of the ability of the different serogroups to cause mouse lethality showed a significant difference between *E. coli* O4 and O18a,c: the former exhibited a significantly higher toxicity ($p < 0.001$). Mouse lethality of all the other serogroups was strikingly lower than that of *E. coli* O4 (O20, $p < 0.001$; O75, $p < 0.001$; O115, $p. < 0.01$). The ability to cause mouse lethality in the different serogroups decreased as follows: O18a,c, O6; O115; O147, O75, 020.

Out of the 158 strains those belonging to *E. coli* O19, O25, O26, O28a,b, O32, O51, O53, O55, O73, O78, O79, O83, O105, O111, O112, O114, O117, O119, O124, O136, O142, O145, and O148 serogroups uniformly failed to show lung toxicity. None of the nine strains belonging to *E. coli* O143 serogroup killed the mice except strain 85170 which, as demonstrated in Table I, was pathogenic. In Table II the incidence of mouse lethality is also presented. These data revealed that mouse lethality was low in *E.coli* serogroups O20 and O75. In the case of *E.coli* O124 and O143 that are capable to penetrate epithelial cells, guinea pig eye test was also performed. In spite of the fact that 6 out of 12 *E.coli* O124 and 5 out of 9 *E.coli* O143 strains caused keratoconjunctivitis, none of these cultures proved to be pathogenic to mice.

Table II

Lung toxicity of E. coli serogroups analysed by their year of isolation, origin and haemolytic activity

E. coli serogroup(s)	Total	Year of isolation				Origin of strains			Haemolysis	
		1974	1975	1976	1977	Faecal sample		Extra-intestinal infection	Positive	Negative
						Enteritis	Healthy			
O4	46/83* 111/450**	5/11 6/55	11/30 20/150	0 0	30/42 85/245	26/49 69/280	1/7 2/35	19/27 40/135	46/65 111/370	0/18 0/80
O18	16/54 38/275	1/12 1/60	12/29 18/145	2/3 18/20	1/10 1/50	8/27 25/145	4/15 7/70	4/12 6/60	16/21 38/105	0/33 0/170
O20	1/29 1/145	0/17 0/85	0/7 0/35	1/3 1/15	0/2 0/10	1/22 1/110	0/2 0/10	0/5 0/25	1/3 1/15	0/26 0/130
O75	3/47 3/235	1/24 1/120	1/12 1/60	0/3 0/15	1/8 1/40	2/34 2/170	1/3 1/15	0/10 0/50	3/8 3/40	0/39 0/195
O115	3/13 4/65	2/10 3/50	1/3 1/15	0 0	0 0	3/11 4/55	0 0	0/2 0/10	3/3 4/15	0/10 0/50
O147	1/14 2/70	0 0	1/14 2/70	0 0	0 0	0/1 0/5	1/13 2/65	0 0	0 0	1/14 2/70
O6	1/1 1/5	1/1 1/5	0 0	0 0	0 0	0 0	0 0	1/1 1/5	1/1 1/5	0 0
Other***	0/158 0/790	0/52 0/260	0/59 0/295	0/25 0/125	0/22 0/110	0/127 0/635	0/14 0/70	0/17 0/85	0/2 0/10	0/156 0/780
Total positive	71/241 160/1245	10/75 12/375	26/95 42/475	3/9 19/50	32/62 87/345	40/144 101/765	7/40 12/195	24/57 47/285	70/101 158/550	1/140 2/695
Total examined	71/399 160/2035	10/127 12/635	26/154 42/770	3/34 19/175	32/84 87/455	40/271 101/1400	7/54 12/265	24/74 47/370	70/103 158/560	1/296 2/1475

* Upper row: No. of toxic strains/total No. of strains

** lower row: No. of died mice/No. of infected mice

*** O19, O25, O26, O28a, b, O32, O51, O53, O55, O73, O78, O79, O83, O105, O111, O112, O114, O117, O119, O124, O129, O136, O142, O143, O145, O148

Discussion

Recently a new method has been described based on early death after intranasal inoculation of albino mice for testing the pathogenicity of certain *E.coli* strains [22, 23]. In some respects this test differs from that of VOINO-YASENETSKY and KHAVKIN [21] showing the bronchoepithelial affinity of mice to shigellae. The lethal effect of bacteria is due to their toxic material [22, 23].

In a premature ward of one of our hospitals an epidemic of enteritis necrotisans with fatal outcome occurred. *E.coli* O4 : K3 was isolated from the stools and umbilical swabs of premature infants and at autopsy. The strains showed the same antibiotic sensitivity and phage pattern; they failed to produce enterotoxin and caused no keratoconjunctivitis of the guinea pig eye. The virulence for albino mice was usually low (LD_{50} 10^6), as compared to the more virulent strains originating from septic cases (LD_{50} usually between 10^4–10^5 [6]).

Lung toxicity test was carried out with 3 strains and all of them caused within 4 hr fatal haemorrhagic pneumonia in 18 out of 25 mice.

KÉTYI *et al.* [23] called for further investigations to elucidate the pathogenic role of the toxin. Examining a large number of strains from clinically well-defined material representing different serogroups, our aim was to detect (i) loss of virulence on storage; (ii) correlation between pathogenicity and serogroup; (iii) parallelism between lung toxicity and other tests for proving the pathogenicity; (iv) relationship between lung toxicity and human pathogenicity.

(i) Although strains recently isolated were more toxic for mice than laboratory strains, the time of maintenance did not influence mouse toxicity significantly.

(ii) Thirty-two *E.coli* strains and eight other facultatively enteropathogenic strains were examined. The incidence of mouse lethality was the highest in serogroup *E.coli* O4 besides *E.coli* O18a, c. In serogroups O20, O75, O115 and O147 mouse lethality was less frequent or the number of strains examined (O6) was not sufficient for drawing unequivocal conclusions. The data mentioned above based on 26 serogroups and 72 strains were in accordance with those of KÉTYI *et al.* [23].

(iii) There was no correlation between enterotoxicity, epithelial penetration, mouse virulence and lung test. These results confirmed those of AVDEEVA *et al.* [22] who failed to find a parallelism between shigella keratoconjunctivitis and lung test. Accordingly, the lung test is not associated with epithelial affinity.

Nevertheless, a close correlation was found between the haemolytic activity of *E.coli* in extraintestinal infections [2] and mouse toxicity of the

strains, in agreement with the results of KÉTYI *et al.* [23]. The discrepancy in haemolytic activity of some strains might have been due to the use of different methods.

Our results were compared with the clinical background, i. e., the source of the strains *E.coli* O4 and O18a,c most frequently responsible for mouse lethality. There was no reliable relationship between the clinical course and mouse pathogenicity.

It may be assumed that, at the present stage of our knowledge, a positive lung test cannot be considered a criterion of the aetiological role of strains isolated in the course of an epidemic.

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THE ADHERENCE OF STAPHYLOCOCCUS EPIDERMIDIS TO HUMAN TONSIL LYMPHOCYTES*

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Attachment of various bacteria to human, peripheral blood and tonsil lymphocytes was investigated *in vitro*. About 20% of tonsil lymphocytes bound *Staphylococcus epidermidis*, whereas the binding of other strains was negligible. The influence on cytoadherence of human serum, immunoglobulins (human IgM, IgG, IgA, as well as their respective anti-Ig's), and carbohydrates (mono and polysaccharides) was measured. It was found that heterogeneous surface structures of the lymphocytes participate in the attachment.

The attachment of bacteria to lymphoid cells has been observed and exploited for the demonstration of antibody producing cells *in vitro* [1–12]. Cytoadherence is the result of a specific reaction which occurs between the surface antibody of the lymphoid cells and the bacterial surface antigen.

Later, bacterial adherence to a variety of mammalian cells has been described [13–21]. It has been reported that cells of various bacterial species attach to B or T lymphocytes, while other species attach to both; *Brucella melitensis* attached to B cells only, and *Bacillus globigii* was bound exclusively by T cells while *Corynebacterium xerosis* attached to both [22–24].

The mechanism by which bacteria bind to animal cells is not known in detail.

In our experiments bacterial adhesion to human tonsil lymphocytes was investigated using different human saprophytic and pathogenic bacterial strains. The percentage of bacterium-binding lymphocytes was assessed by light microscopy. The results suggested that in addition to immunoglobulins, certain carbohydrate groups of the lymphocyte membrane participate in the mediation of cytoadherence.

Materials and methods

Preparation of lymphocytes. Tonsillar and peripheral blood lymphocytes were used. Surgically removed tonsils of children 3 to 6 years old were minced in cold TC 199 medium. The cells were washed 3 times then resuspended in the same medium. Peripheral blood lymphocytes were prepared from defibrinated human blood diluted with phosphate buffer saline

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(PBS). Lymphocytes from either source were purified by centrifugation on Ficoll–Uromiro gradient. The cells were collected from the interphase then washed twice with TC 199. The granulocytes were removed with carbonyl iron powder [25]. Gradient centrifugation was repeated to obtain the lymphocyte suspension (5×10^6 cells/ml).

Separation of T and B cells. The method of JULIUS *et al.* [26] was applied using nylon wool (Leuco-Pak) column.

Bacterial strains. Bacillus cereus 101004, *Brucella abortus* 93002, *Escherichia coli* 30156, *Corynebacterium xerosis* 132006, *Proteus vulgaris* 60001, *Staphylococcus aureus* 110002, *Staphylococcus epidermidis* 110001, *Streptococcus lactis* 80146, were obtained from the Hungarian National Collection of Medical Bacteria, Budapest, Hungary. *Escherichia coli* 02, *Staphylococcus epidermidis* 04, *Staphylococcus aureus* 06, *Staphylococcus salivarius* 05, were obtained from routine specimens of human origin (the numbers are our own designations). *Staphyloccus aureus* Cowan I was a gift by R. VARRÓ, Human Institute for Serobacteriological Production and Research, Budapest.

Preparation of bacterial suspension. The bacteria were grown in nutrient broth (Difco) at 37 °C, with shaking. After washing 3 times with PBS, the bacteria were suspended in PBS to 10^{10} organisms/ml. Heat inactivation of staphylococci was performed at 56 °C for 4 hr. The loss of viability was controlled on nutrient agar plates. Some batches of cocci were treated with trypsin (50 μg/ml) at room temperature for 30 min, followed by washing 3 times with PBS.

Preparation of antibody coated cells. Different classes of Ig, were bound by glutaraldehyde to bacteria according to the method of TEODORESCU *et al.* [22]. The same Ig's were adsorbed to lymphocytes by the procedure of KUMAGAI *et al.* [27].

Assay of adherence. The cells were mixed to a final concentration of 5×10^6 lymphocytes/ml and 1×10^8 bacteria/ml, final volume 4 ml. The suspension was kept at room temperature for 15 min unless otherwise stated, then centrifuged at 100 g for 6 min. The cells were washed 5 times with TC 199, then smears were prepared and stained with Löffler's methylene blue. The slides were examined under a light microscope. A minimum of 400 lymphocytes with or without adhering bacteria were counted.

Measurement of binding of immunoglobulins to cocci. IgM, IgA or IgG (400 μg/ml) was added to cocci (2×10^8/ml). The mixture was kept at room temperature for 20 min then centrifuged. The amount of bound globulin was calculated from the decrease in the protein concentration of the supernatant [28].

Chemicals were of the highest grade avaible. The Ig's were purchased from the Human Institute for Serobacteriological Production and Research, Budapest.

Results

Binding of different bacteria to tonsil and peripheral blood lymphocytes. Stationary phase microorganisms were used in the assay of adherence. Preliminary experiments showed that the counting of rosettes (lymphocytes surrounded by bound bacteria) was not disturbed by free bacteria when the original ratio of bacteria to lymphocytes was 30 : 1. Rosettes contained up to 50 bacteria; then mean value was 22 as determined by the examination of 370 rosettes. Lymphocytes surrounded by 5 or less bacteria were not regarded as rosettes. It was possible to differentiate between lymphocytes and other cell types (monocyte, macrophage, epithelial cell) in stained smears. This was of special importance in the case of peripheral blood lymphocyte preparations which also contained 8–15% polymorphonuclear cells capable of bacterium binding. Lymphocytes were accompanied by 4% contaminating cells (monocytes, plasmocytes) [29, 30]. Binding of cells of the examined bacterial species (cf. Methods) to tonsillar lymphocytes was as follows: *E. coli* 02, 4%; *S. aureus* 110002, 6%; *S. aureus* Cowan I, 11%; and *S. epidermidis* 04, 19%. At the same

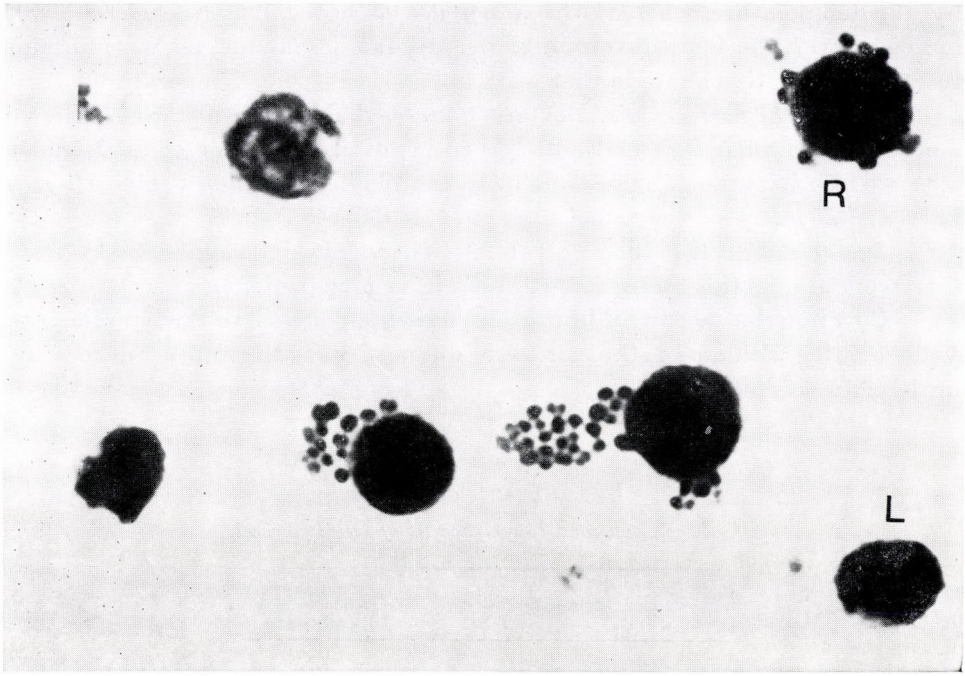

Fig. 1. Attachment of *S. epidermidis* 04 to tonsillar lymphocytes. ×1600. R: rosette; L: lymphocyte without bound bacteria

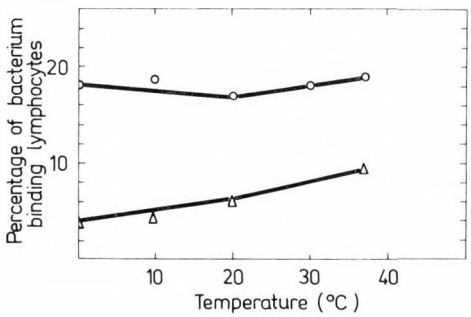

Fig. 2. Effect of temperature on cytoadhesion. Tonsillar lymphocytes were mixed with native (o) or heat killed (Δ) *S. epidermidis* 04 cells. Cytoadherence was assayed as described in Methods

time, binding to peripheral blood lymphocytes was 4% for *S. aureus* 110002, and 5% for *S. epidermidis* 04. The other species did not attach to either type of lymphocytes. The adherence of *S. epidermidis* 04 to tonsil lymphocytes (Fig. 1) was investigated in detail.

Effect of temperature on the adherence of native and heat-killed cocci. Adherence of *S. epidermidis* 04 to tonsillar lymphocytes was assayed at different temperatures (Fig. 2). The binding of native cocci was practically unaffected

by the temperature, whereas the adherence of heat-killed *S. epidermidis* 04 increased with the temperature. Significantly more lymphocytes were forming rosettes with native bacteria than with killed cocci at any temperature.

The role of surface Ig's. Bacteria were coated chemically with different monospecific human Ig's and anti-Ig's to investigate the role of the lymphocyte surface immunoglobulins (sIg's) in the binding reaction (Table I). It is known that many of the tonsil lymphocytes (36–64%) carry sIg's; (IgG 13–39%, IgM 17–22%, IgA 13–37% [31–33]. The values determined in this institute were also in this range [ANTONI *et al.*, in preparation].

Covering the cocci with Ig prevented their adherence to the lymphocytes, while coating with anti-IgG or anti-IgM did not reduce the number of rosettes; anti-IgA was inhibitory.

Table I

Attachment cocci to lymphocytes of Ig-coated cocci

Coating Ig	Percentage of bacterium-binding lymphocytes
Native	19
Human IgG	0
Human IgM	0
Human IgA	0
Anti-human IgG	18
Anti-human IgM	16
Anti-human IgA	3

Table II

Attachment of cocci to Ig-treated lymphocytes

Pretreatment with	Percentage of bacterium-binding lymphocytes
Native	19
Human IgG	12
Human IgM	4
Human IgA	14
Anti-human IgG	35
Anti-human IgM	34
Anti-human IgA	7

In another experiment the lymphocytes were incubated with monospecific Ig's and anti-Ig's (passive coating). After removal of the non-bound Ig, the lymphocytes were mixed with cocci (Table II). IgM inhibited the adherence, while anti-IgG and anti-IgM increased the number of rosettes. Anti-IgA was inhibitory.

Effect of human serum. Ten per cent pooled human Rh^+ serum completely inhibited the formation of rosettes. Pretreatment of lymphocytes with serum (at 0 °C for 30 min) also prevented the binding reaction.

Table III

Attachment of cocci to T and B cells

Cocci	Percentage of bacterium-binding lymphocytes	
	T	B
Native	6	28
Heat-killed	5	15

Adherence to T and B cells. Table III shows the difference between binding ability of T and B subpopulations separated by nylon wool. B cells were not only more active in the binding of native bacteria but they seemed to be responsible for the difference observed in the binding of native and killed cocci.

Binding of Ig's to staphylococci. Ig's were to inhibit adherence, therefore we investigated the binding of Ig's by bacteria. *S. aureus* Cowan I, known to bind IgG by surface protein A [34], was used as control (Fig. 3). *S. epidermidis* 04 bound much IgM but also IgA and IgG. Heat-treatment reduced the IgA binding by about 50%, whereas it increased IgM binding markedly. Pretreatment with trypsin did not alter IgA and IgG binding by the cocci, but increased the binding of IgM.

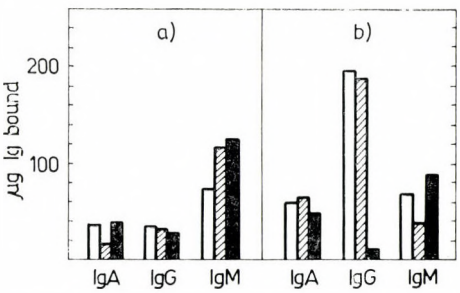

Fig. 3. Binding of immunoglobulins to *S. epidermidis* 04 (a) and *Staphylococcus aureus* Cowan I (b). Clear columns: native; shaded columns: heat-killed; solid columns: trypsin treated cocci

The Cowan I strain showed a high IgG binding ability; IgA and IgM were also bound. Heat-treatment changed the IgM binding only. Trypsin treatment reduced IgG binding by about 90%, in accordance with the results of VIRGILIO *et al.* [35]. The binding of IgA and IgM was hardly affected,

Inhibition of cytoadherence by monosaccharides and materials used for preparation of lymphocytes. With regard to the possible involvement of surface carbohydrates in adherence, we investigated the attachment in the presence of different monosaccharides and complex carbohydrates used for the preparation of density gradients applied in the isolation on lymphocytes (Table IV).

The binding of bacteria was inhibited by galactose (40 mg ml^{-1}) and mannose (20 mg ml^{-1}); Uromiro reduced the adherence drastically.

Table IV

Cytoadherence in the presence of monosaccharides and materials used for preparation of lymphocytes

Agent	Contrentration, mg ml^{-1}	Percentage of bacterium-binding lymphocytes
None	—	19
D-Glucose	5	18
	40	19
D-Fructose	5	19
	40	17
D-Galactose	5	18
	20	17
	40	14
D-Galactosamine	5	17
	40	19
D-Mannose	5	18
	10	17
	20	14
	40	8
EDTA[1]	0.2%	18
Ficoll[2]	2%	16
Uromiro[3]	15%	2
Lymphoprep[4]	2%	17

[1] ethylenediamine tetraacetic acid, tetrasodium salt

[2] Pharmacia (sopolymer of sucrose and epichlorohydrine)

[3] Bracco (solution of methylglucamine and sodium diatrizoate)

[4] Pharmacia (mixture of sodium metrizoate and Ficoll)

Discussion

S. epidermidis 04 was shown to attach *in vitro* to nearly 20% of human tonsil lymphocytes but not to peripheral blood lymphocytes. Modification of the binding revealed several characteristics of the cytoadherence reaction.

1. The specific nature of the binding reaction is supported by the finding that the attachment was not influenced by temperatures between 0 and 37 °C.

2. Heat-treatment seemed to alter or damage the surface structure of bacteria resulting in a decreased, temperature sensitive attachment. Residual binding was probably due to some heat-resistant component.

3. Several, heterogeneous surface structures of the cocci and lymphocytes are involved in rosette formation, as suggested by the effects of immunoglobulins and carbohydrates. Staphylococci have been amply investigated from the immunological point of view. Surface proteins, polysaccharides, mucopeptides and capsular material have been identified as antigenic determinants, and the activation of peripheral blood lymphocytes by cocci has also been described [36–43]. There is, however, no indication that some antigen–antibody reaction is underlying the phenomenon of rosette formation of lymphocytes with cocci.

As regards the lymphocytes, different receptors are considered to be responsible for the binding of antigens, antigen–antibody complexes, complement/immune complex, heterologous red blood cells and lectins [44]. Surface immunoglobulins are likely candidates for the binding of bacteria. This is supported by the predominance of B cells and the relative inactivity of T cells in cytoadherence reactions (Table III). Human serum inhibited the attachment, similarly to earlier findings [45]. The percentage of lymphocytes reacting with anti-IgG and anti-IgM-covered cocci was close to the amount of bacterium-binding lymphocytes. It is therefore supposed that surface IgG-like and IgM-like structures are responsible for the binding of cocci. It is likely that not only the number but also the orientation of surface Ig's is important, since cocci are known to carry Fc receptors. Addition of anti human IgG and human IgM to the lymphocytes may produce a surface rich in free Fc parts, which can react with cocci, leading to enhanced cytoadherence (Table II). Another indication of the importance of the orientation of Ig's is that IgM adsorbed onto the surface of lymphocytes inhibited the attachment. In this case the Fc part of the molecule was connected to the surface of lymphocytes, and it was not available for the binding of bacteria, which were shown to fix IgM (Fig. 3).

A comparison of the Ig binding of *S. epidermidis* 04 and *S. aureus* Cowan I containing protein A revealed a marked difference (Fig. 3). The former bacteria did not show high IgG binding abolished by trypsin treatment, as found with the latter strain. We could not explain the effect of heat on the binding of Ig's by *S. epidermidis* 04. The binding of IgA and IgM by the Cowan I

strain did not dissppear after trypsin treatment. This finding supports the suggestion of HARBOE and FÖLLING [46] that protein A is not responsible for the binding of IgM. The existence of several types of receptors for the binding of different Ig classes has been assumed earlier [47–49]. The results shown in Table IV suggest that, in addition to immunoglobulins, other surface structures of the lymphocytes also participate in the binding of bacteria. The blocking effect of different carbohydrates indicated the possible involvement of carbohydrate containing molecules. Bacteria have been shown to attach to non-lymphoid human cells, too; certain carbohydrate groups of surface glycoproteins participate in this linkage [50]. We found that mannose strongly inhibited cytoadherence, therefore it is supposed that a mannose-specific, lectin-like structure of the bacterial surface may react with some mannose containing polysaccharide or glycoprotein of the lymphocyte surface.

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ESTIMATION OF VIBRIO CHOLERAE AND ESCHERICHIA COLI HEAT-LABILE ENTEROTOXIN BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)*

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By Enzyme-Linked Immunosorbent Assay (ELISA) purified cholera and *Escherichia coli* enterotoxins can be detected as sensitively as by CHO cells. There is a linear relationship between toxin concentrations and extinction values. In plates sensitized with toxins, antitoxins can be titrated with high sensitivity. ELISA cross-titration experiments demonstrate the existing antigenic relationship between cholera toxin and heat labile *E. coli* enterotoxin. Plates sensitized with either anti-*E. coli*-IgG or anti-cholera-IgG are suitable for detecting both cholera toxin, and *E. coli* LT. ELISA seems to be a simple, sensitive and economic method for quantitation of enterotoxins and toxin-specific antibodies.

In the last few years we have compared different techniques [1] for evaluating enterotoxins, to find a simple, sensitive screening method for toxin detection. Since in the Virus Laboratory of this Institute ELISA has successfully been applied for detecting both viral antibodies [2] and alpha-fetoprotein [3], we decided to adopt the method also for quantitation of enterotoxins and their antitoxins.

The study was aimed to clarify the parameters essential for standardizing the technique. Purified or semipurified toxins and antitoxins were used and their interaction was studied in homologous and heterologous combinations.

Materials and methods

Enterotoxins. Purified cholera toxin (CT) prepared by Dr. R. A. FINKELSTEIN (Lot. No. 4493) was obtained from Dr. I. Joó (Human, Budapest); in some cases we used the Wellcome Toxin (VT 2210L5, Wellcome Laboratories, Beckenham, England). Semipurified heat labile enterotoxin of *E. coli* (LT) was kindly provided by Dr. F. DORNER (Sandoz Forschungsinstitut, Wien). Stock solutions of toxin preparations were kept at 4 °C.

Antitoxins. Anti-cholera toxin (Swiss Serum and Vaccine Institute, Bern) was purchased it was of horse origin having the nominal values 36 000 loop U/ml and 10 000 PF U/ml.

Anti-*E. coli*-LT serum and anti-cholera toxin were raised by immunizing rabbits with purified Dorner LT and Finkelstein CT, respectively, according to CALLAHAN *et al.* [4]. A mixture of toxin and complete Freund adjuvant (v/v) was injected subcutaneously. At one week intervals 3 further doses were given, and one week later 3 doses of toxin were given intravenously at 4-day intervals.

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Bleeding was done one week after the last injection. Sera were filter-sterilized and kept at −20 °C.

Antitoxins were purified for IgG by the standard method [5]. IgG fractions and alkaline phosphatase (Sigma, type VII) were coupled by glutaraldehyde. Rabbit anti-LT-IgG, anti-CT-IgG, anti-rabbit-IgG, and anti-horse-IgG were coupled with horse-radish peroxidase by Dr. R. VARRÓ (Human, Budapest).

Cell culture. Chinese hamster ovary (CHO) cells were kindly provided by Dr. B. ROWE (Central Public Health Laboratory, London). They were maintained in 50 cm^2 Roux flasks in MEM medium completed with 10% newborn calf serum and transferred weekly by trypsin-EDTA detachment. For testing toxins, 2-or 3-day-old cultures were used.

CHO elongation test was performed in 96-well Linbro tissue culture plates. Hepes-buffered MEM (50 µl/well) was added to each well and then a 50 µl amount of toxin solution was given to the first well and twofold dilution was done. Then about 5000 cells in 50 µl medium were added to each well, and the plates were covered and incubated overnight at 36 °C. The toxic effect was evaluated by microscopic examination. Instead of determining the actual percentage of elongated cells [6] we considered as titre of the toxin the highest dilution in which 30 to 50% of cells showed typical elongation regarded as 1 CHO elongation unit (CHO-E).

Neutralization in CHO cells. In these experiments 2–6 CHO-E units of toxin were used. First, antisera were diluted into the wells, then to every dilution an equal volume (50 µl/well) of toxin was added. The mixtures were incubated at 36 °C for 45 min when cells were added to the wells. After overnight incubation at 36 °C, evaluation was done. The highest dilution of a given antiserum which still neutralized the elongating effect of the toxin was accepted as titre. On every plate serum controls and toxin controls (retitration) were set up, the actual amount of toxin was determined according to the result of retitration.

ELISA Technique. 1. For toxin detection Dynatech Microelisa plates were coated with antibodies : (anti-CT diluted 1 : 500 (horse) and 1 : 5000 (rabbit) or anti-LT diluted 1 : 30 000. For antitoxin titration, plates were sensitized with antigens (CT diluted 1 : 200 or LT diluted 1 : 300); 100 µl of toxin or antitoxin was added to each well and the plates were kept overnight at 4 °C.

2. From different dilutions of antigens or antibodies 100 µl amounts were added to the wells of the sensitized plates. Incubation was done for one hour at 30 °C.

3. After incubation, the plates were washed three times with PBS-Tween (0.1 M of PBS pH 7.2, containing 0.05% Tween 20). Then appropriate conjugate at the predetermined optimal dilution (100 µl) was added and the plates were incubated as above.

4. After washing, 150 µl of the chromogens (p-nitrophenyl-phosphate for alkaline phosphatase and 1,2-phenylendiamine-hydrochloride for peroxidase) were added. With alkaline phosphatase substrate the reaction time was one hour, while with peroxidase it was 30 min at 30 °C.

5. The enzyme reaction was stopped with 50 µl/well of 3 M NaOH for phosphatase and with 50 µl of 4 M H_2SO_4 for peroxidase.

6. *Evaluation.* The interaction between enzyme and substrate results in a yellow colour for phosphatase and brown for peroxidase. The colour intensity measured by a photometer is proportional to the amount of antibody (antigen) attached to the sensitized plate. A Dynatech ELISA Reader was used for registering absorbances: phosphatase activity was read at 405 nm, peroxidase activity at 492 nm.

Results

Detection of cholera toxin, E. coli LT and their antitoxins in homologous system. First the effect of different conjugates was determined on the reproducibility of the test. In plates sensitized with anti-CT-IgG, by the use of antibody of alkaline phosphatase-conjugated anti-CT-IgG as second antibody, CT could be detected with high sensitivity. Repeating these experiments five times, one month apart comparable results were obtained (Table I). Extinction (measured at 405 nm) $\geq$0.15. was considered a positive reaction. So, a 1 : 1600

Table I

Detection of cholera toxin in plates sensitized with anti-cholera IgG (horse)

Reciprocal dilutions of cholera toxin	Extinction values, experiment					Mean extinction value
	1.	2.	3.	4.	5.	
20	1.50	1.50				1.50
40	1.20	1.30				1.25
80	1.00	1.10				1.05
100			1.00	1.00	1.00	1.00
160	0.84	0.93				0.88
200			0.68	0.86	0.84	0.79
320	0.58	0.66				0.62
400			0.48	0.60	0.58	0.55
640	0.39	0.41				0.40
800			0.35	0.41	0.31	0.35
1280	*0.21*	*0.23*				0.22
1600			*0.16*	*0.21*	*0.18*	*0.18*
3200			0.08	0.08	·	0.08

Cholera toxin: Finkelstein Lot No. 4493. Anti-cholera-IgG (horse) was diluted 1 : 300; as second antibody, alkaline phosphatase-conjugated anti-cholera-IgG was used at 1 : 300 dilution. Substrate reaction time was 1 hr at 30 °C. Extinction $\geq$0.15 were considered specific (figures printed in italics)

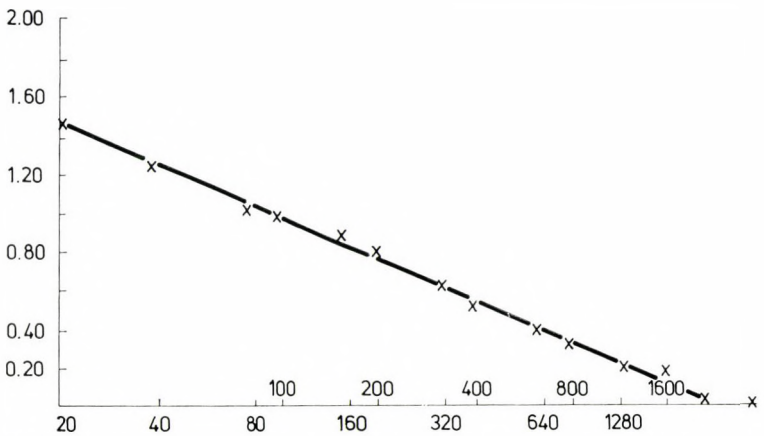

Fig. 1. Relationship between concentration and extinction. Titration of cholera toxin in plates sensitized with anti-CT-IgG

dilution of CT (stock solution) was accepted as titre of the toxin. Since the stock solution had a nominal value of 1800 Lb, the sensitivity of the assay was about 1.12 Lb/ml. The same stock solution gave a CHO titre of 1 : 512 (3.5 Lb/ml).

Thus, the relative sensitivity of ELISA was comparable to that of the CHO elongation test.

Reproducibility of ELISA was indicated by the similar extinction values obtained at different points of time. The relationship of extinctions and dilutions showed a linear dose-response curve (Fig. 1).

Similar results could be obtained when plates were sensitized with a 1 : 30 000 dilution of anti-LT-IgG. In such plates Dorner LT could be detected at a dilution of 1 : 80 000, in accordance with the 1 : 76 800 titre in the CHO test (Table II). In these experiments we compared two types of conjugate. Results showed that the enzyme part of the conjugate had no role in the test's sensitivity (Table II).

Table II

Detection of coli LT in plates sensitized with anti-LT-IgG by the use of two conjugates

Dilution of LT (Dorner) (log10)	Extinction values obtained with	
	anti-LT-IgG/AP (405 nm)	anti-LT-IgG/HRPO (495 nm)
10^{-2}	1.95	>2.00
10^{-3}	0.76	0.83
10^{-4}	0.46	0.31
2×10^{-4}	0.28	0.27
4×10^{-4}	0.20	0.21
8×10^{-4}	*0.15*	*0.16*
16×10^{-4}	0.09	0.12

Anti-IgG was diluted 1 : 30 000. Alkaline phosphatase (AP)- and horse radish peroxidase (HRPO)-coupled anti-LT-IgG were used at 1 : 1000 dilution. Extinction values $\geq$0.15 were considered specific (figures printed in italics)

Next, plates were sensitized with the toxins to titrate antitoxic antibodies. When plates were sensitized with *E.coli* LT, the homologous antibody titre was 1 : 32 000, showing a linear association between concentration (dilution) and extinction (Fig. 2). In this case, peroxidase labelled anti-rabbit IgG (diluted 1 : 1000) was used as second antibody. Results are presented in Table III. It is difficult to compare this titre with that of the CHO neutralization test; in the latter, anti-LT-serum neutralized 6 CHO-E at a dilution of 1 : 1600.

The results showed that ELISA can effectively be used for evaluating purified enterotoxins, with a sensitivity comparable to that of the CHO elongation test. The technique is especially advantageous for titrating antibodies; depending on the toxin dose used it is about 20 times more sensitive than the CHO neutralization test.

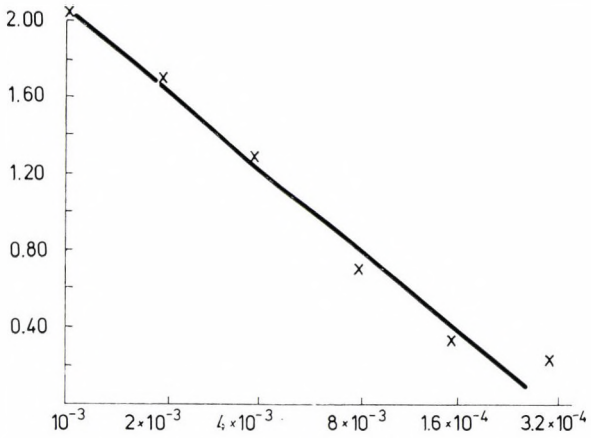

Fig. 2. Relationship between concentration and extinction. Titration of anti-LT-serum in plates sensitized with *E. coli* LT

Table III

Titration of E. coli LT antitoxin on plates sensitized with E. coli LT (Dorner)

Dilution of antitoxin (log10)	Extinction value (492 nm)
10^{-3}	$\geq$2.00
2×10^{-3}	1.70
4×10^{-3}	1.30
8×10^{-3}	0.66
1.6×10^{-4}	0.33
3.2×10^{-4}	0.23
6.4×10^{-4}	*0.09*

E. coli LT was diluted 1 : 300; as second antibody, anti-rabbit IgG/HRPO was used at 1 : 1000 dilution. Extinction $\geq$0.15 was considered specific (figures printed in italics)

Antigenic relationship between LT and CT. The aim was to clarify whether the antigenic relationship of cholera toxin and *E.coli* LT [7–12] could be utilized for titrating heterologous toxins by ELISA. In order to obtain standard values, cross-neutralization tests were carried out in CHO cell cultures. Results of a representative experiment are shown in Table IV. In the test we applied equal doses of toxins (6 CHO-E), these were neutralized with homologous and heterologous antitoxins. Though heterologous titres were lower than the homologous ones, the results indicated an antigenic relation between LT and CT.

To investigate cross-reactions by ELISA, we sensitized plates first with the antigens. Since anti-*E.coli* LT-serum was raised in rabbits, peroxidase-

Table IV

Neutralization of cholera and E. coli enterotoxin with homologous and heterologous antitoxins in CHO cell culture

Toxins	CHO-E*	Highest dilution of sera neutralizing elongation	
		anti-LT	anti-CT (horse)
LT	6	1 : 1600	1 : 200
CT	6	1 : 400	1 : 400

* CHO elongation unit

conjugated anti-rabbit-IgG was used for detecting antibodies. For measuring cholera toxin antibodies as the anti-CT serum was of horse origin, alkaline phosphatase-conjugated anti-horse-IgG was used. Results obtained in homologous and heterologous combinations are shown in Table V; they agreed well with those obtained in the CHO neutralization test (Table IV). Heterologous titres reached 25 to 50% of the homologous ones, indicating again the strong antigenic relationship between CT and LT.

Table V

Homologous and heterologous titres of anti-CT and anti-LT sera by ELISA

Toxins	Reciprocal titres of antitoxins	
	anti-LT	anti-CT
LT	32 000	1000
CT	16 000	4000

Plates were sensitized with the toxins. For anti-LT, as second antibody, anti-rabbit-IgG/HRPO, for anti-CT, as second antibody, anti-horse-IgG/HRPO was used at 1 : 1000 dilution

ELISA allows to test the antigenic relation in a reverse way, too, when plates are sensitized with the antibodies. In this system LT and CT were titrated against homologous and heterologous antibodies (Table VI). In plates sensitized with anti-LT-IgG, CT could be detected in a titre reaching 50% of the homologous one. On the other hand, when the plate was sensitized with anti-CT-IgG of rabbit serum, LT could only be detected at an equally high dilution (1 : 3200).

Table VI

Homologous and heterologous titres of cholera and E. coli enterotoxin by ELISA

Toxins	Reciprocal titres of toxins	
	anti-LT	anti-CT (rabbit)
LT	2000	3200
CT	1000	3200

Plates were sensitized with the toxins. For anti-LT, as second antibody, anti-rabbit-IgG/HRPO was used

Discussion

Several *E.coli* strains [13, 14] as well as other enteric bacteria [15—17] like salmonellae [18, 19], *Shigella flexneri* [20] etc. are capable of producing enterotoxins. The enterotoxins produced are frequently LT or antigenically related to LT.

A routine diagnostic method for detecting enterotoxins is lacking, mainly because of the technical difficulties. The majority of the tests used for detecting LT [6, 21–25] is not applicable for routine purposes. We have found that the blueing test [1] might be used routinely, though the sensitivity of animals differs widely. Cell culture techniques are promising tools, but they are expensive, require a special laboratory and sterile filtrates without preservatives.

An economic screening technique would therefore be needed for both diagnostic and research work. The former work is complicated by the occurence of enterotoxigenic strains in healthy persons [26] and by the fact that the quantity of LT produced varies greately [27]. So, apart from the possible role of adhesion factors, it is not known what LT level may be accepted as indicative for potential pathogenicity. Thus, it would be important to have a screening method of high sensitivity and a rapid, sensitive test for studying immune responses to enterotoxins.

Purified CT and LT of good antigenicity are now available and they may be utilized for developing simple serological techniques. For determining cholera toxin, passive immuno-haemolysis [28] or the passive reverse haemagglutination test [29] can be used, but they are not recommended for large scale screening.

Since ELISA had been developed [30] it was used in cholera serology [31, 32] and recently for detecting LT. In a study [34], gangliosid G_{M1}, a receptor for both toxins [33], was used as sensitizing agent. With this G_{M1}-ELISA, receptor bound LT could be detected with a somewhat lower sensitivity than in Y-1 adrenal cell culture. YOLKEN *et al.* [35] detected LT with the

use of ELISA in polystyrol plates. In performing the test, they utilized the antigenic relationship between CT and LT.

Our experiments were aimed primarily at studying the sensitivity and applicability of Micro-ELISA. For this technique, it was important to have purified LT and CT.

With Dynatech Microelisa plates and Elisa Reader, it was possible to use ELISA for measurement of enterotoxins and antitoxins.

In a homologous system ELISA detects toxins (LT or CT) with a sensitivity equal to that of CHO cells. The technique is especially sensitive in evaluating antitoxins. With photometric evaluation, ELISA can be standardized for assaying crude toxins and also for measuring antitoxic immunity.

In antitoxin titration, ELISA proved to be more sensitive than the CHO neutralization test. It detected the antigenic relationship between CT and LT at the same rate as did the CHO neutralization test. In LT titration, plates sensitized with heterologous (CT) and with homologous (LT) antitoxins showed the same degree of sensitivity for detecting toxins.

In summary, once ELISA has been standardized with purified toxins and antitoxins, the test can effectively be used for assaying toxins and toxin-specific antibodies. For reliable evaluation, photometric reading is required.

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MEMBRANE CHARACTERISTICS OF THE *hem*A MUTANT OF BACILLUS SUBTILIS

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(Received May 17, 1979)

The membrane of the δ-aminolaevulinic acid synthase (EC. 2.3.1.37) deficient mutant of *Bacillus subtilis* growing in the presence of δ-aminolaevulinic acid differs only to a limited extent from the wild type. In haemin-containing medium, however, significant differences are observed as regards the osmotic stability of the protoplasts and the membrane protein composition.

In bacterial cell membranes many biochemical and biophysical events occur. The most important are the biosynthesis of the cell wall and lipids, electron transport, and oxidative phosphorylation. The membranes of the porphyrin auxotrophs may be different from those of the prototrophs. Some proteins are absent in *Rhodopseudomonas spheroides* mutants with blocks in bacteriochlorophyll synthesis [1].

One group of *Bacillus subtilis* porphyrin mutants (*hem*A) are δ-aminolaevulinic acid (ALA) synthase-deficient. These bacteria are not able to produce ALA, the first compound in the porphyrin biosynthetic pathway. They grow only in the presence of ALA or haemin and other additional materials [2].

This paper describes experiments carried out to determine the protoplast stability and membrane protein content of the ALA synthase-deficient strain growing in two different media.

Materials and methods

Bacterial strain. Bacillus subtilis trp C2 *hem*Al [2].

Lysis curves. The strain was grown in HAC [3] medium, or in gGM [2] containing ALA (2 μg/ml) at 37 °C with shaking. After reaching OD 1.0, the cells were centrifuged and washed, and grown for 3 hr in gGM supplemented with tryptophan (50 μg/ml; starvation). Thereafter the culture was centrifuged and washed with 0.7 M SP (0.7 M sucrose, 0.05 M Na_2HPO_4, 0.01 M $MgCl_2$ pH 7.0) buffer, and the pellet was resuspended in 0.8 ml 0.7 M SP buffer and 0.2 ml of lysozyme (10 mg/ml in SP buffer). The suspension of cells in lysozyme was incubated at 37 °C for 30 min to convert the cells to protoplasts. The conversion was followed by phase contrast microscopy. Samples of protoplast suspensions were then diluted tenfold with 0.05 M Na_2HPO_4–0.01 M $MgCl_2$ containing sucrose, so that the final concentration of sucrose ranged from 0 to 0.7 M. After incubation for 10 min at 37 °C the optical density was determined at 620 nm. The method was a slight modification of that of Kent *et al.* [4].

Preparation of membranes for gel electrophoresis. The mutant was grown as described above. At the late logarithmic phase the cultures were harvested by centrifugation. The pellets

were washed twice with 0.6 M SP buffer and resuspended in 1 ml of 0.6 M SP buffer. The membrane preparation was carried out as described [4].

SDS-polyacrylamide gel electrophoresis. The membranes were prepared for electrophoresis by treatment of 150–250 μg of membrane protein with a solution of 3% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, and 40 m M Tris HCl (pH 8.0). The mixture was heated in boiling water for 1.5 min [1]. Electrophoresis was carried out by the method of LAEMMLI [5].

Results

Lysis curves. To determine quantitatively the osmotic fragility of the mutant growing in the media containing ALA or haemin, its lytic behaviour in suspension was studied. After being grown in the presence of haemin, protoplasts of the mutant were much less stable (Fig. 1). When bacteria were

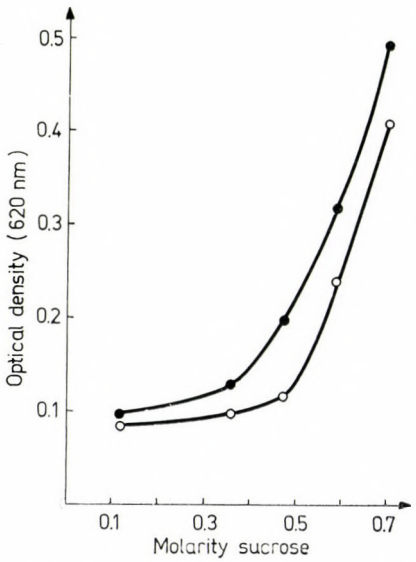

Fig. 1. Lysis curves of protoplasts. The *hem*A mutant was grown in HAC (○), or ALA-containing (●) media

grown in the presence of ALA, the protoplast stability approximated that of the wild-type strain.

Membrane protein composition. There were significant differences in the membrane protein composition, too. As can be seen in Fig. 2, some protein bands are absent or are present in a very limited quantity when the mutant is grown without ALA. The growth is slower in HCA medium. We assume that other, more significant, differences would be observed on longer starvation, but no longer starvation period was applied, because the sporulation of these mutants is very fast under the given circumstances. In the presence of ALA

a biosynthesis of corrins and porphyrins takes place in these mutants. Every enzyme containing corrin or porphyrin structure is formed; hence, in ALA-containing medium only a small difference can be observed between the mutant and wild-type strain.

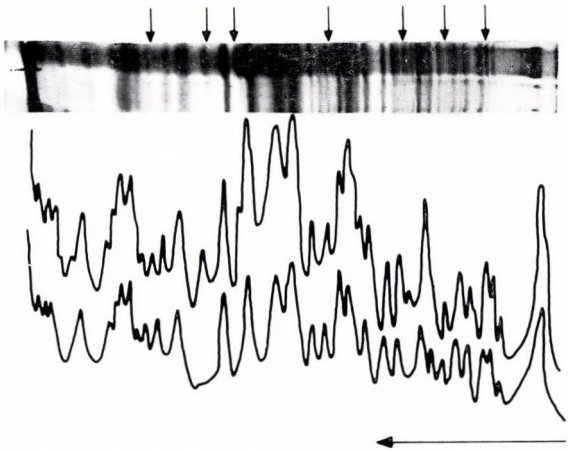

Fig. 2. SDS-polyacrylamide gels of membranes of *hem*A mutant grown in HAC (lower), or ALA-containing media (upper), and densitometer scans of the stained gel

Discussion

Mutation in prophyrin biosynthesis results in changes in the membrane structure. When the *hem*A mutant is grown in the presence of ALA, membrane stability and protein composition are similar as in the wild type, even after starvation. In haemin-containing medium more differences occur (Figs 1, 2). In this case more membrane protein components are absent or modified. The enzymes containing prophyrins and mainly corrins are present at low concentrations [6].

TABER *et al.* [7] found some differences in the membrane protein composition of the menaquinone-deficient *B. subtilis* mutants, which were cytochrome-deficient, too.

More detailed comparisons are premature since, to our knowledge, no further information is available concerning membrane protein patterns in energy-deficient mutants of *B. subtilis*.

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YERSINIA ENTEROCOLITICA INFECTION OF ANIMALS AND HUMAN CONTACTS

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(Received May 15, 1978)

Infective animal sources and persons in contact with animals were investigated for *Yersinia enterocolitica*. The 68 isolates from 3115 animals belonged to four serogroups. The most frequent was serogroup O3 (62 strains) found mainly in pigs (41 strains). The same serogroup was isolated from dogs (9 strains), cats (6 strains) and from other animals (6 strains). A total of 556 animals living in the Budapest Zoo were examined. *Y. enterocolitica* was isolated from the faecal specimens of one chimpanzee and one gibbon. Using the Widal-type agglutination, out of sera of 877 persons in contact with animals, 4.56% were positive with antigens O3 and O9, compared to 0.33% in healthy controls. The study indicates that beside the occasional role of other animal species, pigs should be regarded as the main source of human enteric yersiniosis.

The increase of *Yersinia enterocolitica* isolations from human clinical material has directed attention to the obscure epidemiological characteristics of yersiniosis. Yersinia infection can cause manifest disease in man and animals or they can become symptomless carriers. Among the possible sources of human infection, mainly the role of domestic animals, small rodents and other free-living animals has been subject to investigation.

In 1970, in the Netherlands Esseveld and Goudzwaard [1] isolated 53 *Y. enterocolitica* strains from feacal specimens of 641 pigs with diarrhoea kept at 223 farms of which 15 proved to be infected. In another series they isolated 19 *Y. enterocolitica* strains from 138 healthy pigs bred at 4 farms. In 1972, in Belgium, 14 of the 28 animal strains isolated by Vandepitte *et al.* [2] originated from pigs, the others from cows, hares, chinchillas and canaries. In 1976, in Denmark, Pedersen [3] cultivated 17 strains (7 belonging to serogroup O3) from the bowel contents of 100 pigs at the slaughterhouse. In another series, the microorganism was isolated from 12 of 222 pigs with various diseases and from 3 of 40 diseased dogs. In 1973, in Japan, Tsobokura *et al.* [4] reported on the cultivation of 13 strains from the bowel contents of 299 healthy pigs. In 1974, the Japanese authors Zen Yoji *et al.* [5] examined faecal and lymph node specimens of healthy slaughterhouse pigs and cattle. Isolation of *Y. enterocolitica* was successful from 231 of the 2173 pigs but from none of the cattle. Rats trapped at the slaughterhouse also proved to be infected though the serogroups of the isolates differed from those causing human or

porcine infections. BEFEKADU *et al.* [6] isolated *Y. enterocolitica* of a different serogroup from the pet dog of a diseased child. In 1973, in Finland and Sweden, AHVONEN *et al.* [7] examined faecal and necropsy specimens from 3057 healthy or diseased domestic animals. Of the 7 strains, 4 were isolated from dogs, 2 from pigs and 1 from cats. In 1974, in Canada (Ontario, Alberta and British Columbia) TOMA and LAFLEUR [8] isolated 22 strains from domestic animals (dog, cat) and other animal species (beaver, washbear, camel, Canada goose, chinchilla, oyster). In 1974, ALDOVÁ and LIM [9] collected 104 samples from 446 rodents trapped in three areas of Bohemia. They isolated 60 strains, the serogroups differed from area to area. In 1975, in France ALONSO and BERCOVIER [10] isolated 4 strains from rodents captured in two regions of the country lying distant from each other. In 1976, ALONSO *et al.* [11] reported on investigations including 1906 small rodents (mouse, field mouse, wood mouse, shrew, mole). They isolated 70 strains and some others from fish caught in fish ponds within the trapping area. In the studies of KAPPERUD [12] conducted in 1975 in Norway and Sweden, 25 *Y. enterocolitica* strains were isolated from 551 small rodents (6 species) trapped at six localities. The isolates were of different serogroups. The same author [13] examined rodents and mammals in Norway and Denmark. *Yersinia* was isolated from the faeces of 31 of 305 small rodents and from 5 of 31 shrews. Three of 25 red foxes harboured *Y. enterocolitica*. KAPPERUD and JONSSON [14] isolated 3 strains from 3 of the 20 examined brown trouts. In 1977, POKORNÁ and ALDOVÁ [15] isolated from sewer rats *Y. enterocolitica* O3. In 1972, OLÁH *et al.* [16] in Hungary investigated domestic animals living in the environment of persons with various diseases and isolated *Yersinia* on several occasions. In 1973, KUBINYI (unpublished data) isolated from the faeces of 22 pigs, and SZITA and SVIDRÓ [17] from the faeces of 2 pigs and of a hen *Y. enterocolitica* in the course of an investigation made because of a family outbreak.

The studies have demonstrated that different serogroups of *Y. enterocolitica* strains can be isolated from various animal species [18] but there has been no documented evidence whether regular contact with animal reservoirs can be made responsible for human disease or latent infection.

The present paper reports on studies aimed at establishing the occurrence of *Y. enterocolitica* infection among various domestic animal species and among wild animals kept in the zoo, further among persons in regular contact with domestic animals.

Materials and methods

A total of 2559 faecal specimens from the animal stock of different cooperative farms, from slaughterhouses and small private farms was examined. The number of mammals and bird studied in the Budapest Zoo amounted to 556. Only a minority of the material originated from animals living in the environment of diseased persons, since OLÁH *et al.* [16] have already reported on this kind of observations. The lymph nodes of 69 pigs, 30 chicken embryos and

30 batches of feed were cultured on desoxycholate citrate agar plates. Part of the material was enriched in Wauters's medium; later in the course of the study enrichment was not applied.

The strains grown in culture were identified biochemically and serologically as described in a previous paper [19]. Widal-type agglutination with O3 and O9 antigens as reported earlier [20] was performed using the sera of 1500 blood donors, sera of 877 persons living in contact with animals, and 2192 patients' sera sent to the laboratory for diagnostic purposes. The mean titres of the sera were compared.

Results

Table I shows the distribution of *Y. enterocolitica* strains according to the animal species and the percentual occurrence of the serogroups. Four serogroups: O3, O4, O5 and O10 were differentiated. A total of 68 strains could be obtained by culture from the 3115 specimens. Most of them originated from pigs (41 strains), dogs (9 strains) and cats (6 strains). The cats lived in the environment of diseased persons and were symptom-free according to the data available. *Y. enterocolitica* could be isolated from other animals too, as shown in Table I. In the faeces of the symptomless chimpanzee and gibbon, the microorganism was present in pure culture. Most (91.1%) of the strains belonged to serogroup O3 responsible in Hungary for most of the human infections. No strains could be cultured from lymph node, chicken embryo and animal feed specimens.

Table I

*Incidence of Y. enterocolitica in animals in Hungary**

Animal species	Number of samples	Serogroups, number of strains				Total number of strains
		O3	O4	O5	O10	
Pig	1209	41				41
Cat	13	6				6
Dog	50	9				9
Fowls	314	3 (hen)	1 (duck)	2 (hen, duck)		6
Cattle	330	1		1		2
Sheep	463			1		1
Hare	4	1				1
Animals in zoo	556	1 (chimpanzee)			1 (gibbon)	2
Other	176	—				—
Total	3115	62	1	4	1	68
Per cent		91.1	1.5	5.9	1.5	100.0

Table II shows the results of serological examinations. The sera gave a positive reaction with the two antigens (O3 and O9) in 0.33% of the donors, in 4.56% of the persons in regular contact with animals, and in 26.6% of those

suspected of suffering from enteric yersiniosis. In the same order of succession the mean titres for antigen O3 were 1:15.4, 1:31.9, 1:214.8, and for antigen O9 1:15.3, 1:18.5, 1:76.4. The higher titres with antigen O3 and the lower ones with antigen O9 are in agreement with the higher and lower incidence of these serogroups in man and animals [21].

Table II

Titre of sera from different groups of persons against Y. enterocolitica antigens O3 and O9

Reciprocal serum dilutions	Healthy persons		Persons in contact with animals		Patients	
	O3	O9	O3	O9	O3	O9
<10	1348	1445	723	822	1391	2163
10	78	32	29	18	47	2
20	57	15	50	28	71	5
40	15	5	37	7	96	6
80	1	3	20	1	106	7
160	1	0	16	1	112	5
320	0	0	2	0	97	4
640	0	0	0	0	75	0
1280	0	0	0	0	68	0
2560	0	0	0	0	64	0
5120	0	0	0	0	63	0
10240	0	0	0	0	3	1
Mean titre*	15.4	14.3	31.9	18.5	214.8	76.4
Positive sera, per cent	0.33		4.56		26.60	
Total number of sera	1500		877		2193	

* Mean titre $= \sqrt[N]{d_1^{n_1} \cdot d_2^{n_2} \ldots d_n^{n_n}}$

$d_1\ d_2 \ldots d_n$ = reciprocals of serum dilutions

$n_1\ n_2 \ldots n_n$ = number of sera giving the corresponding titre

N = total number of sera

Discussion

Epizootics caused by Y. *enterocolitica* were reported in 1961–1963 in chinchilla farms in Switzerland, the Netherlands and the German Federal Republic [22], followed by the first diagnosed human enteric yersiniosis cases in Europe. MOLLARET [23] maintains that the date is so important that two

periods should be distinguished in the history of yersiniosis, one before and one after 1962. It seems that the infection was imported to Europe from North America. The first two *Y. enterocolitica* strains originated from chinchillas dead while shipped from Canada to Denmark, the other from a chinchilla dead in Finland several months after it had been imported from the USA. Subsequently epizootics broke out in the chinchilla farms of Denmark and Zurich and become widespread in the German Federal Republic, the Netherlands and Belgium; smaller outbreaks were reported from Sweden and France. Nevertheless, THAL [24] believes that *Y. enterocolitica* as an animal pathogen is of limited importance. ALONSO *et al.* [18] have listed several animal species serving as reservoirs for the microorganism. In our material, the isolates from chimpanzee and gibbon complement their data.

The majority of our isolates were obtained from pigs. A transport of a cooperative farm to the slaughterhouse yielded positive cultures in 50%. Beside the 3 strains obtained from animals living in the zoo, 7 *Salmonella* strains were isolated. In a series including 398 pigs, beside the 10 *Y. enterocolitica* strains, 15 *Salmonella* types were demonstrated using selenite enrichment. Our findings are in agreement with those of ESSEVELD and GOUDZWAARD [1], further with those of ZEN YOJI *et al.* [5] who also suggest that pigs have an important role in human yersinia enteritis. This is supported not only by the identical serogroups dominating in human infections, but also by the seasonal incidence of isolations as reported by ZEN YOJI *et al.* [21]. We suppose that seasonality may be related to the period when pig-killing is customary. VAN OYE [25] emphasizes the connexion between pig breeding in West Flanders and human yersiniosis cases in contrast to North France where pig-killing is not a custom thus no human diseases occur. MOLLARET *et al.* [26] analysing the same fact found that infection with *Y. enterocolitica* is 200 times more common in Belgium than in France and this is the case also in the border area. Whereas between 1967 and 1974, 153 strains were isolated in Belgium mainly from pigs, only 60 were identified in France mainly from hares. The border between the two countries is politicial and not ecological therefore it is hard to find an explanation to this paradox, especially because the demographic and nutritional factors are similar. No notable differences were found as concerns the incidence of salmonellae in the border area.

The Widal-type reaction introduced by WINDBLAD *et al.* [27] and recommended by others [28] proved to be well suited in the diagnostics of *Y. enterocolitica* and in the detection of latent infection of persons in contact with infective sources. Serum specimens from persons in contact with animals (mainly pigs) were positive in 4.56%, whereas from healthy persons only in 0.33% that is 13.8 times of the controls. Our material does not include the 80 slaughterhouse workers whose sera were examined every 3 months and were positive in 15.0%. The high positive titres are supported by the fact that 2 of the 10 persons with

latent infection suffered from gastroenteritis at the time of the examination. Humans can become infected through contact with cats and dogs, too. Nine strains belonging to the serogroup O3 were isolated from dogs, 6 strains from cats. As keeping dogs as pets is common, a connexion with the infection of children can justly be suspected.

Our results and literary data indicate that infection with *Y. enterocolitica* occurs in man and animals. Various animal species (fish, birds, wild mammals, rodents, domestic animals) can excrete different serogroups of the microorganism and be symptomfree. Strains of varying antigenic structure can be demonstrated also from surface waters. Man living in close contact with the animal reservoirs are easily infected and harbouring the strains can excrete the agents without being actually ill. The question arises whether yersiniosis can be regarded as an anthropozoonosis. The question is not easy to answer. If we think of the *Y. enterocolitica* strains belonging to a number of serogroups and carried by various animals, it seems plausible that infection with *Y. enterocolitica* is actually a saprozoonosis, i.e. the common disease of man and animals, the reservoir of which is the environment [22].

From among the animal reservoirs, a special epidemiological relationship exists between pig and man. The frequency of the serogroup O3 in human and animal (mainly porcine) specimens, the antibody titres indicating latent infection of persons in contact with animals (mainly pigs) suggest that domestic animals, primarily pigs, play an important part as sources of human infection, therefore the epidemiological role of pigs may regarded as orthozoonostic.

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ECOPHYSIOLOGICAL ANALYSIS OF A BACILLUS MEGATERIUM POPULATION IN A BROWN FOREST SOIL

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From the A_F, A_H and B horizons of a brown forest soil in Hungary, 447 bacterial strains were isolated on a non-selective basis. One hundred and sixty (36%) of the isolated strains proved to be *Bacillus*, and of these 143 (32%) were identified as *Bacillus megaterium*. The occurrence of this species is more or less restricted to the A_F and A_H horizons, where its local population is considerably homogeneous from the diagnostic point of view. Thirty-three representatives of this *B. megaterium* population were examined in detail. All of these strains are similar in some markers different from the typical *B. megaterium*. This local population is described as *Bacillus megaterium* var. *thermotolerans* var. nov. Some actual problems of the ecology of *B. megaterium* are discussed in the light of the present findings.

Species of the genus *Bacillus* are widespread in every geographical region, and in almost every horizon of different soil types. They are represented by species of highly different biotic potentials in terrestric soils as well as in muds and subhydric soils of the coastal area of rivers and lakes. In the last decade this genus has become the focus of multisided ecological–taxonomical research work [1–7]. Special attention has been paid to *B. megaterium*, a species regarded as an indicator organism of favourable soil properties. MISHUSTIN and MIRZOEVA [2] suggested that in many soils the degree of mineralizing activity and of the energetical degradation of organic materials in natural soils is correlated primarily with the local population density of *B. megaterium*. The same authors believe that this species might be used as an indicator organism of the fertilitity of agricultural soils. Unfortunately, little is known of the soil-geographical occurrence of the different species of the genus *Bacillus* including *B. megaterium*. Besides, some publications are based on unsatisfactory diagnostic identifications.

In the present report, the *Bacillus* population of the A_F, A_H and B horizons of a brown forest soil (Man and Biosphere Model Area) is described. We investigated the taxonomical composition, the ecophysiological characteristics and the total platable counts of the bacilli in different soil horizons. Finally, we describe a new variety of the only predominant species of the *Bacillus* population of this model area, denominated *B. megaterium* var. *thermotolerans* var. nov.

Materials and methods

Isolation of bacterial strains. Soil samples were collected under aseptic conditions from the MAB model area near Síkfőkút (Hungary). Samples were taken from the A_F, A_H and B horizons of the brown forest soil. The samples were cooled and processed within 24 hr. Separately-growing colonies were obtained on nutrient agar and synthetic agar plates after incubation at 28 °C for 2–4 days. Colonies were transferred without selection onto nutrient agar slants.

Composition of culture media. Nutrient agar: meat extract (Oxoid), 3 g; peptone (Difco), 5 g; agar, 15 g; distilled water, 1000 ml; pH 7.0. Synthetic medium [8]: glucose, 10 g; $(NH_4)_2HPO_4$, 4 g; NaCl, 5 g; $K_2HPO_4 \cdot 3\ H_2O$, 2 g; $MgSO_4 \cdot 7\ H_2O$, 1 g; $CaCl_2$ 0.4 g; $FeSO_4 \cdot 7\ H_2O$ 0.02 g; $MnSO_4 \cdot 7\ H_2O$, 0.01 g; agar, 15 g; distilled water, 1000 ml; pH 7.0. The strains were maintained either on nutrient agar or on a yeast extract–glucose agar [9]. Composition of the latter: yeast extract, 5 g; peptone, 5 g; glucose, 10 g; agar, 20 g; distilled water, 1000 ml; pH 7.2.

The diagnostic and ecologic–physiologic characteristics studied were as follows.

(1) Cell shape and size in stained smears prepared from 24 hr cultures grown on yeast extract–glucose agar medium.

(2) Gram stain. Twenty-four-hour cultures grown on yeast extract–glucose agar were stained; decoloration in 96% ethanol.

(3) Staining of spores. Malachite green steaming as described by SCHAEFFER and FULTON [10].

(4) Growth at pH 5. The degree of turbidity in nutrient broth was read after 2 weeks incubation.

(5) Growth on synthetic medium in the presence of $(NH_4)_2HPO_4$ as the sole nitrogen source.

(6) Utilization of citrate on Difco Simmon's Citrate Agar.

(7) Hydrogen sulphide production tested with the lead acetate indicator paper described by MORSE and WEAVER [11].

(8) Methyl red and Voges–Proskauer tests. Cultivation in glucose–phosphate medium, demonstration of acetoin according to BARRITT [12].

(9) Catalase activity. Ten per cent H_2O_2 was dropped onto 48 hr cultures and the intensity and the speed of gas production was observed.

(10) Oxidase activity, tested according to KOVÁCS [13].

(11) Active motility. Twenty-four-hour cultures grown on yeast extract agar [14] and 36 to 48 hr cultures grown in semisolid Bacto Motility Test Medium were observed for motility.

(12) Growth on nutrient agar at temperatures 3–5 °C, 9–11 °C, 37 °C, 45 °C and 50 °C.

(13) Thermal death point. Twenty-four-hour cultures grown in nutrient broth were kept for 10 min in a water bath of 40 °C, 50 °C or 60 °C. Then aliquots of the suspensions were spread on the surface of nutrient agar plates. Appearance of colonies after an incubation of 24–48 hr indicates the survival of heat-treated cells.

(14) Growth in Bacto Anaerobic Agar in the course of one-week incubation period.

(15) NaCl tolerance. Nutrient broth media containing 0, 3, 7 and 10% NaCl were inoculated.

(16) Sensitivity to lysozyme. The effect of 0.001% and 0.04% lysozyme solutions was recorded (SCHLEIFER and KLOOS [15]).

(17) Growth on nutrient agar plates containing 5% or 10% bile salts (Difco Bile Salts) during one week incubation.

(18) Growth during 6 days on Bacto MacConkey agar.

(19) Nitrate reduction during 6 days in Bacto Nitrate Broth. Detection according to COWAN and STEEL [16].

(20) Reduction of methylene blue in a nutrient broth containing 0.002% methylene blue.

(21) Sensitivity to antibiotics and antimicrobial substances. For this purpose Resistest disks (Human, Budapest) were applied on plated cultures. The radius of the inhibition zone was measured after 24 hr incubation.

(22) Oxidative or fermentative degradation of carbohydrates (HUGH and LEIFSON [17]).

(23) Utilization of carbohydrates as sole carbon source on Koser's citrate medium modified by GORDON and MIHM [18]. The results were followed up for 3 weeks.

(24) Indole production in peptone water, in nutrient broth and in nutrient broth containing tryptophan 1 g/litre. Reagent: Kovács's reagent or Bacto Indole Test Strips.

(25) Ammonia production in 6-day peptone-water cultures. Demonstration of ammonia with Nessler's reagent.

(26) α-Amylase activity. Demonstrated with Lugol's iodine solution in 5-day cultures grown on nutrient agar plates containing soluble starch (Difco).

(27) Casease activity. Tested in nutrient agar plates containing Bacto Skim Milk.

(28) Liquefaction of gelatin into Bacto Nutrient Gelatin Medium (COWAN and STEEL [16]).

(29) Lipolytic activity: SIERRA [19].

(30) Lecithinase activity: GORDON *et al.* [6].

(31) Arginine hydrolysis. Ammonia production was detected with Nessler's reagent in 5-day cultures grown in arginine–glucose nutrient broth.

(32) Aesculin hydrolysis: COWAN and STEEL [16].

(33) Detection of urease: CHRISTENSEN [20].

(34) Hippurate hydrolysis: THIRST [21], and HARE and COLEBROOK [22].

(35) Detection of DNase and RNase: JEFFRIES *et al.* [23].

(36) Phenylalanine deamination. The method described in [24] was applied for the phenylalanine–yeast medium recommended by EWING *et al.* [25].

(37) Phosphatase production. Nutrient agar plates containing sodium phenolphthalein phosphate were used as described by COWAN and STEEL [16].

(38) Chitinase activity. Colloidal chitin was prepared as described by LINGAPPA and LOCKWOOD [26]. Degradation of chitin was examined on nutrient agar plates containing chitin (GRIMONT *et al.* [27]).

(39) Degradation of cellulose. Modified WAKSMAN's [8] starch–$NaNO_3$ agar was used as basal medium. Basal medium plates were overlayered with basal medium containing 5% cellulose. The cultures were observed over 4 weeks.

(40) Haemolysin activity. Nutrient agar plates containing sterile bovine blood were used.

Note. Mainly Difco, Oxoid and Merck preparations were used as ingredients in the various media.

Results

Four hundred and forty-seven strains were isolated from the A_F, A_H and B horizons. Of these 160 isolates (36%) proved to be *Bacillus*, and among the latter, 143 (32%) of the total, were identified as *B. megaterium* (Table I).

Table I

Distribution of the isolates

Genetic soil horizon	Isolates		*Bacillus* spp. isolates		*B. megaterium* isolates	
	No.	per cent	No.	per cent	No.	per cent
A_F	170	38	36	8	34	8
A_H	170	38	106	24	100	22
B	107	24	18	4	9	2
Total	447	100	160	36	143	32

Thirty-three *B. megaterium* isolates were examined in detail. According to these representative data, the local population of *B. megaterium* is characterized as follows (Tables II, III, IV and V).

Table II

Markers shared by 143 B. megaterium strains
(Diagnostic markers used in primary grouping)

Shape of cells	rodlike
Cell diameter	1.05–1.40 μm
Cell length	2.49–8.06 μm
Gram staining	variable
Shape of spore	oval
Spore diameter	1.23 μm
Spore length	1.56–2.16 μm
Citrate utilization	negative 70%, positive 30%
Growth on synthetic medium	positive
H_2S production	negative 70%, weakly positive 30%
Catalase	positive
Oxidase	positive
Voges–Proskauer reaction	negative
Methyl red test	negative
Growth at pH 5	positive 53%, negative 47%

The cells are large, 1.05–1.4 μm in diameter and 2.49—8.06 μm in length. The oval spore is in central position. The spore wall is thin, the sporangium is not swollen. Fifty-seven per cent of the strains show active motility. The strains are Gram-variable, catalase positive, do not form indole; Voges–Proskauer reaction negative. Growth on synthetic medium containing glucose + ammonium salt: positive; starch hydrolysis: positive; gelatin liquefaction: positive; acid formation from glucose and mannitol: positive. Citrate utilization is negative in 70% of the isolates. Minimum growth temperature: 3–5 °C. Maximum growth temperature: 50 °C. Haemolytic activity: negative; nitrate reduction to nitrite: positive. In general, growth can be observed in the presence of 3% NaCl; urease activity is positive in 50% of the strains. Hydrogen sulphide production: weakly positive in 30% of the isolates; casein hydrolysis positive in 60%; there is no anaerobic growth; lecithinase activity: negative; all strains are sensitive to 0.04% lysozyme; in the presence of 0.001% lysozyme, some colonies were developing from several resistant cells; oxidase test: positive; MR test: negative; 53% of the isolates show weak growth at pH 5. Thermotolerance: 80% of the strains survive heating at 60 °C for 10 min; all strains grow in the presence of 5% bile salt and 54% also in the presence of 10%; no growth on Bacto MacConkey agar; methylene blue reduction: negative. Sensitivity to the indicated concentrations of antibiotics and antimicrobial agents is presented in detail in Table V. All strains form acid from glucose, mannitol, sucrose, fructose, trehalose and glycerol; 70% of the strains form acid from inulin, 85% from

Table III

Properties of 33 representative strains of B. megaterium var. thermotolerans. I. Morphological and physiological–biochemical markers

Markers	No. of strains tested	Positive		Negative		Variable or ambiguous	
		No.	per cent	No.	per cent	No.	per cent
Lipolytic activity							
Tween 40	33	33	100	0	0	0	0
Tween 60	33	31	94	0	0	2	6
Tween 80	33	32	97	1	3	0	0
Urease activity	33	16	48.5	7	21.2	10	30.3
Gelatin liquefaction	33	30	90.9	3	9.1	0	0
Arginine hydrolysis	33	0	0	33	100	0	0
Starch hydrolysis	33	33	100	0	0	0	0
NaCl tolerance 3%	33	33	100	0	0	0	0
7%	33	0	0	33	100	0	0
Casease production	33	20	60.6	13	39.4	0	0
Aesculin hydrolysis	33	26	78.8	4	12.1	3	9.1
Indole formation	33	0	0	33	100	0	0
Ammonia production from peptone water	33	0	0	31	94	2	6
Haemolysin production	33	0	0	33	100	0	0
Growth on MacConkey agar	33	0	0	33	100	0	0
Growth in the presence of							
5% bile salt	33	31	94	2	6	0	0
10% bile salt	33	18	54.5	15	45.5	0	0
Reduction of methylene blue	33	0	0	33	100	0	0
Nitrate reduction to nitrite	33	33	100	0	0	0	0
Growth at 3–5 °C	10	10	100	0	0	0	0
at 50 °C	33	33	100	0	0	0	0
Tolerance to 60 °C	33	26	78.8	7	21.2	0	0
Cellulase activity	33	0	0	33	100	0	0
Chitinase activity	33	0	0	33	100	0	0
Phenylalanine deaminase	33	0	0	33	100	0	0
Formation of chains	33	33	100	0	0	0	0
Motility	33	19	57.6	13	39.4	1	3
DNase activity	33	31	94	1	3	1	3
RNase activity	33	33	100	0	0	0	0
Hippurate hydrolysis	33	33	100	0	0	0	0

Table III (continued)

Markers	No. of strains tested	Positive		Negative		Variable or ambiguous	
		No.	per cent	No.	per cent	No.	per cent
Sensitivity to lysozyme							
0.04%	33	33	100	0	0	0	0
0.001%	10	10*	100	0	0	0	0
Phosphatase	33	33	100	0	0	0	0
Lecithinase	10	0	0	10	100	0	0
Growth in anaerobic agar	33	0	0	33	100	0	0
Utilization of carbon sources							
glucose	33	32	97	1	3	0	0
lactose	33	0	0	33	100	0	0
mannitol	33	32	97	1	3	0	0
L-arabinose	33	1	3	32	97	0	0
sucrose	33	33	100	0	0	0	0
rhamnose	33	19	57.6	14	42.4	0	0
D-fructose	33	33	100	0	0	0	0
D-galactose	33	5	15.2	27	81.8	1	3
L(−)-sorbose	33	0	0	33	100	0	0
D-mannose	33	15	45.5	17	51.5	1	3
maltose	33	32	97	1	3	0	0
melibiose	33	0	0	33	100	0	0
raffinose	33	22	66.7	9	27.3	2	6
D-sorbitol	33	28	84.8	5	15.2	0	0
dextrin	33	33	100	0	0	0	0
D-fucose	33	3	9.1	23	69.7	7	21.2
adonitol	33	2	6	31	94	0	0
inositol	33	5	15.2	28	84.8	0	0
tagatose	33	0	0	33	100	0	0
dulcitol	33	1	3	32	97	0	0
inulin	33	30	90.9	3	9.1	0	0
salicin	33	30	90.9	3	9.1	0	0
glycerol	33	33	100	0	0	0	0

* A few colonies formed by resistant cells

Table IV

Properties of 33 representative strains of B. megaterium var. thermotolerans. II. Acid formation from carbohydrates

Substrate*	Positive strains		Weakly positive strains		Negative strains	
	No.	per cent	No.	per cent	No.	per cent
Glucose	4	12*	27	82	2	6
Lactose	0	0	0	0	33	100
Mannitol	21	64	8	24	4	12
L-Arabinose	0	0	1	3	32	97
D-Xylose	0	0	7	21	26	79
Sucrose	10	30	20	61	3	9
D-Fructose	9	27	22	67	2	6
Dextrin	0	0	11	33	22	67
Dulcitol	0	0	1	3	32	97
D-Galactose	0	0	0	0	33	100
Inulin	2	6	20	61	11	33
Inositol	0	0	1	3	32	97
D-Sorbitol	0	0	19	58	14	42
Rhamnose	0	0	10	30	23	70
Raffinose	7	21	22	67	4	12
D-Mannose	0	0	0	0	33	100
L(−)-Sorbose	0	0	0	0	33	100
Maltose	4	12	2	6	27	82
Melibiose	0	0	0	0	33	100
Trehalose	13	39	18	55	2	6
Cellobiose	1	3	16	48	16	49
Glycerol	5	15	25	76	3	9
Salicin	0	0	0	0	33	100

* Hugh and Leifson's test

raffinose and a half of them from sorbitol and cellobiose. In the presence of $(NH_4)_2HPO_4$ as nitrogen source each of glucose, mannitol, sucrose, fructose, maltose, dextrin, salicin, glycerol and inulin was utilized as sole carbon source; sorbitol was utilized by 85%, rhamnose and raffinose by 60% and mannose by 50% of the strains. Arginine hydrolysis: negative. Aesculin hydrolysis was positive for 80% of the strains. Ammonia was not formed from peptone water; lipolytic activity: positive. Phenylalanine-deaminase production: negative. Neither cellulose nor chitin was degraded by any of the strains. DNase and RNase production, hippurate activity and the phosphatase test were uniformly positive.

Table V

Properties of 33 representative strains of B. megaterium var. thermotolerans. III. Sensitivity to antibiotics

Antibiotic	Resistant (0 mm)*		Slightly sensitive (0.1–12 mm)*		Moderately sensitive (12–20 mm)*		Very sentitive (20–32 mm)*	
	No.	Per cent	No.	per cent	No.	per cent	No.	per cent
Penicillin (3 IU)	0	0	20	61	8	24	5	15
Oxacillin (10 μg)	0	0	21	64	11	33	1	3
Streptomycin (30 μg)	0	0	27	82	6	18	0	0
Chloramphenicol (30 μg)	0	0	0	0	31	94	2	6
Methicillin (20 μg)	0	0	0	0	22	67	11	33
Oleandomycin (20 μg)	0	0	6	18	27	82	0	0
Tetracycline (30 μg)	0	0	0	0	21	64	12	36
Neomycin (100 μg)	0	0	33	100	0	0	0	0
Gentamicin (20 μg)	0	0	32	97	1	3	0	0
Ampicillin (20 μg)	0	0	0	0	15	45	18	55
Lincomycin (10 μg)	1	3	32	97	0	0	0	0
Cephalosporin (10 μg)	0	0	7	21	21	64	5	15
Polymyxin-B (15 μg)	0	0	33	100	0	0	0	0
Erythromycin (10 μg)	0	0	0	0	26	79	7	21
Sulfadimidine (400 μg)	31	94	2	6	0	0	0	0
Nitrofurantoin (300 μg)	0	0	0	0	30	91	3	9
Chlortetracycline (30 μg)	0	0	0	0	28	85	5	15
Colistin (20 μg)	0	0	33	100	0	0	0	0
Spiramycin (30 μg)	0	0	21	64	11	33	1	3
Kanamycin (30 μg)	0	0	30	91	3	9	0	0
Oxytetracycline (30 μg)	0	0	0	0	32	97	1	3
Pristinamycin (10 μg)	0	0	28	85	5	15	0	0
Carbenicillin (50 μg)	0	0	9	27	19	58	5	15
Co-trimoxazole (25 μg)	29	88	2	6	2	6	0	0
Vancomycin (50 μg)	0	0	16	48	16	48	1	3
Nystatin (100 IU)	33	100	0	0	0	0	0	0
Paromomycin (50 μg)	0	0	33	100	0	0	0	0
Novobiocin (30 μg)	0	0	22	67	11	33	0	0
Nalidixic acid (30 μg)	2	6	31	94	0	0	0	0

* Radius of inhibitory zone minus Resistest disk radius

Discussion

The present results clearly show that the *Bacillus* strains dominant among our isolates are identical with the species *B. megaterium*. However, as regards some markers, our isolates are different from the typical representatives of that species, e.g. 70% of the population of our model area are citrate negative, a property occurring infrequently in other populations.

The description in Bergey's Manual [28] as well as by GORDON *et al.* [6] and SZABÓ [29] qualify the acid production from arabinose as variable. Of our isolates, 97% do not form acid from arabinose.

The highest temperature at which *B. megaterium* is able to grow is unequivocally indicated as being between 35 °C and 45 °C. Our isolates, though weakly, do multiply even at 50 °C.

Unlike typical *B. megaterium* strains, none of the strains isolated in Síkfőkút grew in the presence of 7% NaCl.

B. megaterium in general does not form hydrogen sulphide under laboratory conditions, whereas some of our strains proved to be weak producers. According to KNIGHT and PROOM [30] and GORDON *et al.* [6], *B. megaterium* in general is casease-positive. Of our isolates only 60% showed this activity.

GORDON *et al.* [6] regard this species as Gram-positive, though two strains in their list are indicated as Gram-variable. WHITE [31] qualified the majority of his authentic *B. megaterium* strains as Gram-variable. SZABÓ [29] found the NCTC 7581 and the RIPP A-112 strains of *B. megaterium* Gram-variable. Gram-variable strains occurred among PRIETO's [32] isolates as well. Several authors have studied the Gram staining of *B. megaterium* [33–35]; according to SMITH [35], 59% of his *B. megaterium* strains were Gram-positive in contrast to *Staphylococcus aureus* which is regarded uniformly Gram-positive. The strains of the Síkfőkút *B. megaterium* population all proved to be Gram-variable.

The urease activity of the species was qualified variable by KNIGHT and PROOM [30] and definitely positive by OTTOW [3]. The latter author recommended the use of this property as a key marker in species differentiation. In contrast, 50% of the strains isolated in our model area proved to be urease-negative.

It was already pointed out by MISHUSTIN and MIRZOEVA [2] that like other species of the *Bacillus* genus, *B. megaterium* has different ecotypes. The Síkfőkút population may represent such an ecotype. The Gram-variable, in their majority citrate-negative, members of this population which do not multiply in the presence of high salt concentrations but grow even at 50 °C may be separated from the typical members of the species and designated as *B. megaterium* var. *thermotolerans* var. nov.

According to MISHUSTIN and MIRZOEVA [2], large amounts of *B. megaterium* mainly occur in the soil horizons near the surface, namely, in the upper

region of the humus horizon. The same distribution is shown by our *B. megaterium* population; 24% of the isolates originated in A_F, 70% in A_H, and only 6% in the B horizon. We suppose that in the B horizon *B. megaterium* is a foreign element, passively introduced from upper horizons.

B. megaterium is lacking in most of the northern soils. In MISHUSTIN's opinion [36], its particular pattern of distribution is correlated with the physico-chemical and biological dynamics of the soil. He assumes that *B. megaterium* cannot adapt itself to the climatic conditions of northern regions. At the Liverpool soil-bacteriological symposium, WOLF [37] pointed out that the activating and initiating conditions necessary for the germination of spores of different species are very variable within the *Bacillus* genus, e.g. *B. megaterium* requires glucose for germination. The various soil types offer different ecological conditions for the germination of spores. On the other hand, a *Bacillus* species may die out in a soil or in a milieu in which it cannot sporulate. In the discussion concerning this subject, BARKER [38] pointed out that at unfavourable temperatures some species of the *Bacillus* genus are present in the form of spores while their vegetative forms are lacking. If under such conditions germination is initiated by some stimulus, the vegetative cells will soon die. This may cause the disappearance of *B. megaterium* in northern soils, e.g. in soils of the tundra.

The peculiar dynamics of the dispersal and occurrence of *B. megaterium* is much more complicated. This view is supported by SZABÓ's observations [29]. He found practically no *B. megaterium* in a rendzina soil (Hungary) the plant cover of which contained mediterranean elements. In the bacterial population of this rendzina, another *Bacillus*, *B. subtilis*, was present as a predominant member of the community.

Unfortunately, taxonomical identifications are often based on diagnostic markers insufficient in number. Such uncertainties have led to difficulties in the evaluation of literary data concerning the ecology of *B. megaterium*.

This thermotolerant population of *B. megaterium* is characterized by an intensive extracellular proteolytic activity (gelatinase, casease), a property making able for primary colonization of protein-containing native organic substances, incorporated into the soil. Urea is degraded by half of the strains. All isolates have an enzyme apparatus for lipid degradation, an important property of the pioneer species of zymogenic microfloras. They also utilize a wide scale of carbohydrates and sugar alcohols, a fact convincingly proving the enhanced biochemical potential of *B. megaterium*, which in turn, leads to a high population density of this species in the soil. As sole carbon source, hippurate is utilized by all of our strains and citrate by part of them. These data suggest that *B. megaterium* does not require special growth factors, thus it may remain competitive in the soil after the complex carbon sources have been exhausted.

An ecologically outstandingly advantageous property of our isolates is their growth in the presence of inorganic nitrogen (ammonium salt) as the sole nitrogen source.

Owing to their DNase and RNase activity, they are presumably capable of covering their own nucleotid demands from the nucleic acids of microbial cells disintegrating in the soil and those being adsorbed to soil particles of colloidal size.

Their phosphatase activity represents a further ecological advantage if the inorganic phosphate pool of the soil tends to be exhausted. In this case phosphatase positive strains can mobilize organic phosphorous compounds.

Our *B. megaterium* strains split neither chitin nor cellulose. This is also characteristic of the members of the zymogenic microfloras; after the primary invasion and the colonization of organic substances, and after the exhaustion of the easily degradable materials, chitin and cellulose remain accessible for autochthonous microflora elements possessing more specialized degradative capacities.

The pattern of vertical distribution of *B. megaterium* var. *thermotolerans* in the brown forest soil of the model area of Síkfőkút is in accordance with its obligatorily aerobic nature, which has been proved by the complete lack of its growth in Bacto Anaerobic Agar.

More than 50% of the isolates grew moderately or weakly even at pH 5.0. This tolerance is a significant ecological trait from the aspect of the elution process in the humus horizon of forest soils. The penetrating acidic rain water, being rich in humic acids transported from the uppermost soil layers, attacks the free $CaCO_3$ in the A_H horizon, thus decalcifies the soil matrix and transports calcium into deeper horizons. Moreover, the Ca ions adsorbed in the colloidal complex are exchanged for hydrogen ions. Thus, the humus horizon may acidify either in several loci or *in toto*, and members of the microflora must tolerate the acidity to survive. In the soil profile from which our isolates originate, Ca ions are predominating in the adsorption complex of every horizon, though, owing to the leaching processes, some degree of undersaturation was observed in the complexes of A and B horizons. The concentration of free $CaCO_3$ is e.g. only about 0.5% in the upper horizons in contrast to the 34.1% found in C horizon. Thus, eluviation acts intensely and represents a decisive ecological factor [39].

Our isolates are tolerant to, i. e. are not inhibited in growth by, surface-active agents. They are capable of multiplying within a very wide range of temperature (3–50 °C) and notably tolerate dry heat. In the humus horizon of the forest soil, covered by leaf-litter, soil temperature values exceed 20–25 °C rarely if at all. Consequently, the thermotolerant population of *B. megaterium* easily tolerates even the highest temperatures occurring in the soil.

The intensive growth of our isolates at temperatures as low as 3–5 °C indicates that low temperature is not the exclusive regulating factor in the

distribution of *B. megaterium* in different geographical regions. The occurrence and community position of this species may be influenced by several factors, often in a complex way manifesting itself in the properties of the type of soil. The intensive motility of *B. megaterium* var. *thermotolerans* is a further property that explains the high local population density of this variant in the forest soil of Síkfőkút. Sixty per cent of the isolates showed an active flagellar motility. Unfortunately, at present there are no accurate data on the correlation between the speed of the active motion of *Bacillus* cells *in vivo*, the thickness of the water film covering soil particles and the pF values of the soil.

Obviously, the characteristic water regime of the soil of our model area [39] supplies the optimum milieu for the rapid local dissemination of *B. megaterium*. The salt tolerance of this *B. megaterium* variety is low (3%) but still considerable if we take into account that the uppermost horizons (A_F and A_H) of the brown forest soils are highly permeable and exposed to elution.

The overwhelming majority of the isolates tested in the present work are sensitive to numerous antibiotics and antimicrobial agents (Table V). Such a high sensitivity is characteristic of the genus *Bacillus* in general. It may be attributed to he high antibiotic sensitivity of the members of this genus that, after the organic substances that had supported their rapid growth have been exhausted, they cannot concur even with slowly-growing secondary invaders, *viz.* the antibiotic-producing actinomycetes.

The dominance or codominance of *B. megaterium* in the A_F and A_H horizons of the soil under study suggests that the biodynamics of this soil is very favourable. This view is supported by the particular microstructure of the A_H horizon, which represents a typical "mull"-structure composed of accumulated earthworm excrements of high aggregate stability with a well aerated extensive micro-cave system. It seems that the total count of *B. megaterium* is growing not only in agricultural soils but also in certain forest soil (eutrophic or mesotrophic) types. Supposedly, there is some relationship between earthworm activity and the population density of bacilli. Little is known of the behaviour of *B. megaterium* in the digestive tract of earthworms, though in this milieu the soil microflora undergoes considerable changes both at the species level and in the total population density [40].

Finally, it deserves mentioning that the literary data of the plasmid-bound thermophilism of some *B. megaterium* populations [41] need confirmation.

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INDUCTION OF STABLE L-FORMS OF STAPHYLOCOCCUS AUREUS AND STREPTOCOCCUS FAECALIS

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Stable L-forms were induced from *Staphylococcus aureus* and *Streptococcus faecalis*. These formed typical foamy L-colonies and showed large and small round bodies. They grew continuously on routine antibiotic-free nutrient broth and blood agar media for 12 passages without reversion to their parental forms. At different concentrations of penicillin various morphological forms were observed. Effect of sucrose, normal horse serum and penicillin on their adaptation and stabilization is discussed.

L-forms, being considered potential pathogens, are gaining considerable importance as on the basis of experimental studies [1–3] they may play a role in the pathogenesis of certain diseases [4–10]. They may be induced *in vivo* [11] as unstable or stable L-forms, and, depending upon their type, they may revert back to their parental form or, produce toxins or other metabolites that induce diseases [12–14].

The present study was designed to induce stable L-forms of *Staphylococcus aureus* and *Streptococcus faecalis*, using penicillin as an inducer, and to investigate the effect of inducers and stabilizers on their adaptation and stabilization.

Materials and methods

Bacterial strains. One strain each of *S. aureus* and *S. faecalis* was used. *S. aureus* was isolated in the laboratory from an abscess, *S. faecalis* (B-45-1) was obtained from the Central Research Institute, Kasauli, India.

Induction process. The medium of NIMMO and BLAZEVIC [15] was used; 100 ml of liquid medium (LM) consisted of brain hearth infusion (Difco), 3.7 g; yeast extract (Difco), 10 g; sucrose (Pfizer), 15 g; magnesium sulphate, 0.2 g; normal inactivated mycoplasma-free horse serum, 10 ml; the pH was adjusted to 7.5. The solid medium (SM) was the same but 1.4 g of agar (Difco) was added to it. As an inducer, penicillin G was used at varying concentrations.

To induce L-forms, 0.2 ml of a 24 hr broth culture of the parent bacterium was added to 4.5 ml of LM and incubated for 24–48 hr at 37 °C. After a few blind passages at 48 hr intervals, 0.2 ml was inoculated on SM and incubated at 37 °C for 48 hr. The plates were then periodically examined for the presence of L-forms by light and phase contrast microscopy. Reversion or the ability to grow in the absence of horse serum, sucrose and penicillin was studied following the scheme of MADOFF [16]. At different passage levels, smears were prepared from broth cultures, stained with Giemsa and Gram stains and examined under oil immersion for colonial morphology.

Results

S. aureus L-forms, after 10 passages in LM (penicillin 1000 units/ml), exhibited large and small round bodies with filaments (Fig. 1). After further 7 passages (penicillin 1500 units/ml), these formed typical L-colonies with long filaments and round bodies. After 11 passages, large and small round bodies with central condensations were present. In the centre of the colony, only large bodies were seen with few filaments (Fig. 2). These, however, in penicillin-free medium reverted in a single passage to the parental form, and were therefore further passaged at increased penicillin concentrations (3500 units/ml) and after 9 more passages (total 26) they reverted back in 4 passages. Reversion was gradual. In the passages first Gram-negative, thin filaments appeared, then Gram-negative thin rods, Gram-positive cocci in chains and finally Gram-positive cocci arranged in clusters like bunches of grapes. These were further passaged at 4000 units/ml penicillin and after 5 subsequent passages their reversion and the effect of sucrose and horse serum were studied on their stabilization and adaptation, and their ability to grow. During the passages, these ingredients were gradually decreased. The organisms could still grow in a medium containing 25% of the original amount of sucrose and serum for two passages, and reverted in the 3rd passage. When they were further passaged at 4500 units/ml penicillin, after 19 more passages (a total of 49) they could not revert back even after 12 further passages in sucrose, serum and penicillin-free nutrient broth and nutrient blood agar media.

S. faecalis. After 10 passages (penicillin 1000 units/ml), the L-forms showed large and small round bodies with filaments. The filaments became less

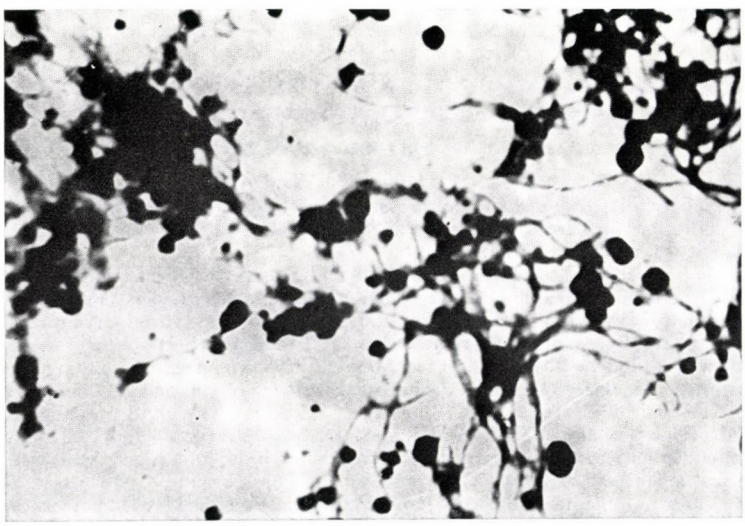

Fig. 1. L-forms of *S. aureus;* filaments and round bodies

and less as the penicillin concentration was increased. At 2000 units/ml of penicillin, large and small round bodies appeared and formed typical foamy L-colonies. In penicillin-free medium they reverted back to parental forms. After 16 additional passages (penicillin 3500 units/ml) they reverted back in 5 passages. When these were further passaged at 4000 units/ml penicillin, after 4 passages the L-forms formed foamy colonies (Fig. 3), with small and large round bodies with coccobacillary forms. These were tested for stability and

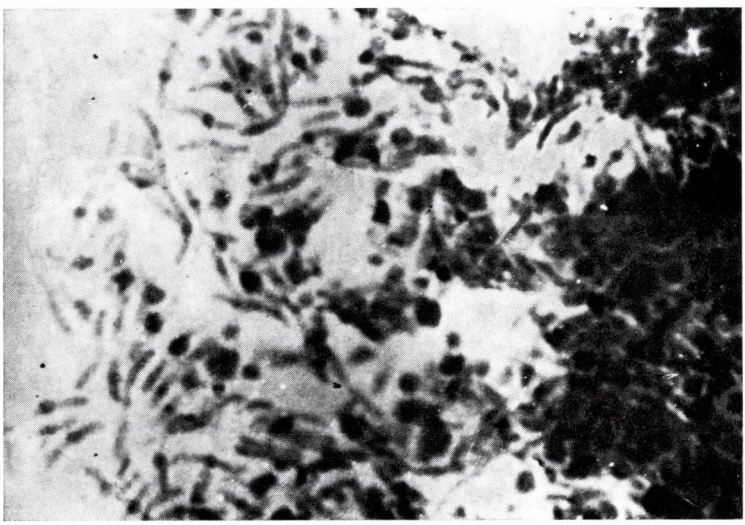

Fig. 2. L-forms of *S. aureus;* large bodies and few filaments

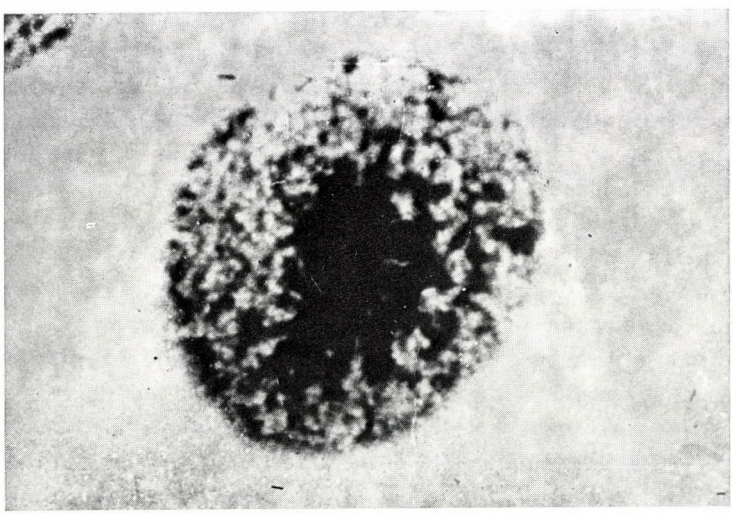

Fig. 3. L-forms of *S. faecalis*

adaptability. They could grow for 2 passages in a medium containing 25% of the original amount of sucrose and serum, and up to the 4th passage in sucrose, serum and penicillin-free medium. They reverted in the 3rd and 5th passage, respectively. If they were further passaged at 4500 units/ml penicillin, after 19 more passages (total 48) they formed typical colonies revealing mostly large bodies. After an additional 4 passages (total 52) they could grow in sucrose, serum and penicillin-free nutrient broth and blood agar media, without reversion even up to 12 passages. The stable L-forms formed typical colonies, thus they were stable L-forms.

Discussion

In the present study, *S. aureus* and *S. faecalis* were used to induce L-forms. Several authors applied different inducers including penicillin to produce L-forms of Gram-positive and Gram-negative organisms [9, 18–28]. In the present study, penicillin was used as the inducer, and sucrose, horse serum and Mg^{++} were applied as stabilizing agents. It was noticed that 500 units/ml of penicillin did not initiate induction and the concentration was increased to 4500 units/ml. Others [15, 29–34] reported that the optimum concentration of inducer varied from species to species to induce these forms from Gram-positive and Gram-negative organisms, while others [11] noticed variations within the species. Some authors [35] used high concentrations of sodium chloride to induce L-forms from staphylococci and produce four types of stable L-forms. Each of these had an optimum concentration of sodium chloride for its growth and failed to grow at higher or lower concentrations.

The stability of L-forms depended upon the concentration of inducer and stabilizers. L-forms produced at 2000 units/ml, reverted back to their parental form in the first penicillin-free passage, thus the reversion was spontaneous. At 3500 units/ml penicillin, reversion was gradual and not spontaneous; the organisms reverted gradually through 4 passages in the case of *S. aureus*, and through 5 passages in the case of *S. faecalis* in penicillin-free medium. L-forms passaged with 4500 units/ml penicillin, on the other hand, not only grew on penicillin-free medium but also in medium free from penicillin, horse serum, sucrose and Mg^{++}.

The stability of L-forms depended besides the penicillin concentration also on the horse serum and sucrose. Various concentrations of sucrose and serum were used to study their effect on the stability and adaptability of L-forms. They did not revert back when the amount of serum was decreased to 50, 25 and zero per cent, keeping the other ingredients constant. Sucrose had a similar effect when reduced gradually. The L-forms still reverted back in the absence of all the three ingredients. MADOFF [16], studying the effect of horse serum, penicillin and sodium chloride on the stability of L-forms of three strains of

Streptococcus MG, reported similar observations, though, the basic medium used was nutrient agar, whereas we used brain heart infusion agar.

The number of passages needed to obtain stable L-forms also varied. MAKAMA and TAKAYOSHI [35] applied 229–333 passages to group A haemolytic streptococci to produce their L-forms. In other study ASNANI (unpublished) used 56 and 100 passages for *Listeria monocytogenes* and *Salmonella typhi*, respectively, to produce their L-forms. Therefore, the number of passages needed to produce these forms, also varied from species to species.

As the penicillin concentration was increased, the filamentous forms decreased and small and large bodies predominated. These were stained darker. Similar morphological forms were also reported [36–39] and these were influenced by the concentration of agar and gelatin in the medium.

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BIOLOGICAL PROPERTIES OF L-FORMS AND THEIR PARENT BACTERIA

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L-forms of *Staphylococcus aureus*, *Streptococcus faecalis*, *Listeria monocytogenes* and *Salmonella typhy* and their parent bacteria were examined for biological properties and compared with their parental forms. Some L-forms differed from their parent bacteria and required a longer incubation period.

L-forms have been isolated from various clinical conditions [1–9], and some experimental studies even suggested their role in various diseases [1, 10, 11]. Data are scarce concerning their biological properties [10–13], especially in comparison with their parental forms. The present project, therefore, was designed to study the biological properties of induced L-forms and to compare them with their parent bacteria so that these could be identified from clinical specimens.

Materials and methods

Source of L-forms. Stable L-forms of 4 pathogenic organisms namely *Staphylococcus aureus*, *Streptococcus faecalis*, *Listeria monocytogenes* and *Salmonella typhi* were induced.

Biological properties. The properties studied included carbohydrate utilization in 1% (w/v) peptone water [14]. To this phenol red indicator (0.2%) and carbohydrates at 1% concentration were added, adjusting the pH to 7.2. The carbohydrates used are presented in Table I. Other properties studied included catalase, oxidase, urease, acetyl methyl carbinol and indole production, citrate utilization, phenylalanine deamination and reduction of nitrates and potassium tellurite [14]. Phosphatase activity and arginine hydrolysis were also studied [15]. Haemolytic activity was studied in blood agar medium. These were also examined for motility wherever necessary.

Results

Results are presented in Table I. The L-forms of the test organisms did not differ significantly from the parent bacterium except in urease activity, nitrate reduction, haemolysin production and utilization of certain carbohydrates, namely mannitol, sucrose, glycerol, rhamnose and dextrin, being negative. These did not show any variation in respect of other biochemical properties except that the incubation period needed was more than that of the parent bacterium namely 48 hr for catalase production, and utilization of citrate, glucose, fructose, lactose, sucrose and mannose and 72 hr for xylose.

Table I

Biological properties of parent organisms and their L-forms

Charact eristics	*S. aureus*		*S. faecalis*		*L. monocytogenes*		*S. typhi*	
	P	L	P	L	P	L	P	L
1. Gram staining	+	−	+	−	+	−	−	−
2. Motility	−	−	−	−	+	+	+	+
3. Haemolysis	+	−	+	−	+	−	−	−
4. Oxidase activity	−	−	−	−	−	−	−	−
5. Catalase activity	+	+	+	+	+	+	+	+
6. Phosphatase activity	+	+	ND	ND	ND	ND	ND	ND
7. Coagulase activity	+	+	ND	ND	ND	ND	ND	ND
8. Urease activity	+	−	ND	ND	ND	ND	ND	ND
9. Nitrate reduction	+	−	−	−	+	−	+	−
10. Tellurite reduction	−	−	−	−	−	−	−	−
11. Phenyl alanine reduction	−	−	−	−	−	−	−	−
12. Indole production	−	−	−	−	−	−	−	−
13. Acetyl methyl carbinol production	−	−	−	−	−	−	−	−
14. Arginine utilization	+	+	+	+	+	+	+	+
15. Glucose	+	+	+	+	+	+	+	+
16. Mannitol	+	−	+	−	+	−	+	+
17. Mannose	+	+	+	+	+	+	+	+
18. Xylose	+	+	+	+	+	+	+	+
19. Fructose	+	+	−	−	+	+	+	+
20. Sucrose	+	−	+	+	−	+	−	−
21. Lactose	+	+	−	−	−	−	−	−
22. Dextrin	+	−	−	−	+	+	−	−
23. Inulin	−	−	−	−	−	−	−	−
24. Sorbose	−	−	−	−	−	−	−	−
25. Rhamnose	+	−	−	−	+	−	−	−
26. Glycerol	+	−	+	−	+	−	+	+
27. Galactose	+	+	+	+	+	+	+	+
28. Salicin	+	−	+	+	−	−	−	−
29. Citrate utilization	+	+	−	−	+	+	+	+
30. Methyl red test	−	−	−	−	−	−	+	−
31. Phospholipids (μg/g), cell wall fraction	24.5	0	29.5	0	23.0	0	19.5	0
32. Phospholipids (μg/g), cytoplasmic fraction	9.5	12.0	11.5	16.0	18.5	21.0	19.5	22.0

ND = not done; P = parent bacterium; L = L-forms

Discussion

L-forms of *S. aureus* differed from their parent bacterium in urease and haemolysin production, nitrate reduction and utilization of mannitol, sucrose, glycerol, rhamnose, and dextrin. They had no haemolytic activity and one of these strains was negative and weakly positive in urease production and arginine hydrolysis, as already described [13]. In respect of acetyl methyl carbinol production, the parent strains were weakly positive, whereas their L-forms were completely negative.

The L-forms of *S. faecalis* did not differ from the parent strains except in haemolysin production, utilization of mannitol and glycerol, being negative for these reactions, whereas the parent bacterium was positive for the above properties. They also required a longer incubation period ranging from 48 to 96 hr as compared to their parent bacterium. Other authors [12] reported strain variations among the strains of *S. faecalis* in respect of the biochemical properties. They studied 3 strains and found that 2 of these showed a weak reaction. All the 3 strains reduced potassium tellurite, whereas their L-forms were weakly positive in respect of this property. Two of these strains oxidized xylose whereas the remaining one did not, whereas we found that L-forms did not vary from the parent bacterium and showed similar biochemical properties.

L-forms of *S. typhi* differed from the parent bacterium as the former neither reduced nitrates nor oxidized dextrin, whereas the parent bacterium did so.

The L-forms of *L. monocytogenes* differed from their parent bacterium as the former did not show any activity in haemolysin, nitrate reduction and utilization of mannitol, glycerol and rhamnose.

Flagellar antigen was reported to be absent in the L-forms of *Vibrio cholerae* and *Proteus vulgaris* [16, 17], whereas others [18] reported its presence. In the present study, L-forms of *S. typhi* and *L. monocytogenes* were motile which suggested the presence of flagella, thus the presence of H antigen. It is well known that the origin of the flagellum is the cytoplasmic membrane of the cell and the cell wall plays no role in its origin, therefore the presence of flagellum in L-forms should, in fact, not be affected. It is evident that probably there is inadequate literature on the comparative study of various biochemical properties of L-forms of these two organisms. We observed that except few variations in some of their biological properties, these did not differ from their parent bacteria significantly.

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PRODUCTION AND ANTIGENIC EFFECT OF EXPERIMENTAL SHEEP ADENOVIRUS VACCINES

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Adenovirus vaccines inactivated and adsorbed with different methods were prepared for the specific control of pneumoenteritis of lambs. Beta-propiolactone and formalin as inactivating agents and aluminium hydroxide gel and DEAE dextran as adjuvants were compared. Vaccines inactivated and adsorbed in different ways provoked practically the same immunological response. Seronegative lambs developed a higher level of antibodies after the administration of monovalent vaccines, than in the case of a bivalent one. It is concluded that inactivated and adsorbed adenovirus vaccines provoke a significant production of antibodies in sheep.

Previously, we have reported on the occurrence of pneumoenteritis of lambs caused by adenoviruses and on the economical importance of the disease [1–3]. The pathogenicity of several local adenovirus strains in experimental infections was also shown [4–6]. Serological surveys revealed a wide occurrence in large farms of all known ovine adenovirus serotypes [7]. These findings called for an active immunization procedure against the disease. In the present paper we report on the production of various mono- and bivalent vaccines and on the immunological response of lambs to vaccination.

Materials and methods

1. *Production of vaccines*

(a) *Viruses.* Virus strains ORT/111, a local isolate of bovine adenovirus type 2 from sheep, and GY/14, a local isolate of ovine adenovirus type 1, were involved in the experiment.

(b) *Multiplication of the virus strains.* Viruses were multiplicated in fetal lamb kidney cultures grown in Eagle's minimum essential medium (MEM, Difco) supplemented with 0.5% lactalbumin hydrolysate and 15% fetal calf serum. As maintenance medium Earle's solution (Difco) was used. The infected 2000 ml Roux bottle cultures were incubated at 37 °C after one hour of adsorption. Growth curves of different strains were preliminary tested [8] and the cultures harvested at optimum time. Optimum virus yield was seen after 40 hr incubation in the case of ORT/111 strain and 64 hr in the case of GY/14 [8]. To release the intracellular virus, the cultures were frozen at −20 °C and thawed three times. Subsequently the virus suspension was titrated and cultures exceeding 10^5 $TCID_{50}$/ml were used for vaccine production.

(c) *Methods of inactivation.* Two methods of inactivation were compared. Cell-free virus suspensions were mixed with serial dilutions of formalin and beta-propiolactone (BPL) to obtain a final concentration from 0.05 up to 0.25%. The samples were mixed and stored at room temperature in the case of formalin and at 4 °C in the case of BPL, for 24 hr. To check inactivation, 6 tubes of fetal lamb kidney cultures of each dilution were inoculated. Residual

formalin was neutralized with sodium metabisulphite and samples were dialyzed against maintenance medium at 4 °C for 24 hr [9].

(d) *Adjuvants.* The potency of alhydrogel (0.75% end concentration Al_2O_3, pH 7.7) and DEAE dextran (7.5%) adsorbed vaccines inactivated with formalin or BPL was compared.

(e)*Preparation of vaccines.* Five experimental vaccines were produced. Monovalent vaccines (Nos 1–4) were prepared from virus strain ORT/111 and a bivalent one (No. 5) from virus strains ORT/111 and GY/14.

Vaccine No. 1. The virus suspension was mixed with aluminium hydroxide pH 7.8 at room temperature for 20 min, then inactivated with formalin pH 7.25 for 24 hr.

Vaccine No. 2. Virus suspension was inactivated with formalin for 24 hr, then mixed with DEAE dextran for a further hour.

Vaccine No. 3. Virus suspension BPL treated at 4 °C for 24 hr was similarly adsorbed on DEAE dextran.

Vaccine No. 4. BPL inactivated virus was adsorbed on aluminium hydroxide.

Vaccine No. 5. A bivalent vaccine (method of vaccine No. 1) was prepared from a 1 : 1 mixture of the two virus strains.

2. *Experimental animals*

Commercial lambs were used in the experiments.

Experiment No. 1. Four groups, each of 8 lambs aged four months were vaccinated with vaccines Nos 1, 2, 3 and 4, applied intramuscularly in the neck (2 ml dose). The vaccination was repeated 3 weeks later. Blood samples were collected on the day of the first and second vaccination and on days 8, 42 and 56 after the first vaccination.

Experiment No. 2. Five lambs aged 8 weeks were vaccinated with vaccine No. 1 twice in an interval of 14 days. Blood samples were taken on days 0, 10, 24, 42 and 56.

Experiment No. 3. Fifteen lambs aged 3 months were vaccinated with vaccine No. 5 on a single occasion. Blood samples were collected on days 0, 14, 28 and 35.

Serum samples were tested against 100 $TCID_{50}$/0.1 ml virus in the neutralization test.

Results

The experiments revealed that formalin and BPL inactivated the virus in a concentration of 0.05 and 0.1%, respectively. According to these findings and for safety, formalin and BPL were used for vaccine production at concentrations of 0.1 and 0.15%, respectively.

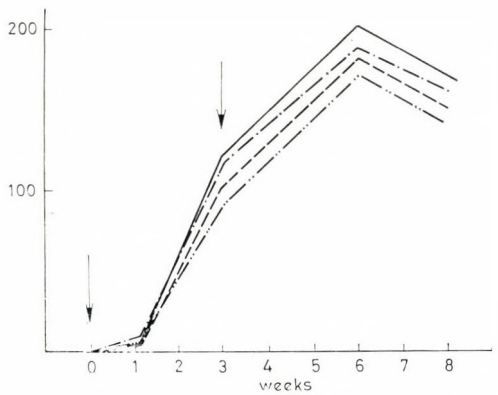

Fig. 1. Dynamics of serological responses following vaccination with different monovalent vaccines (geometric mean titres)

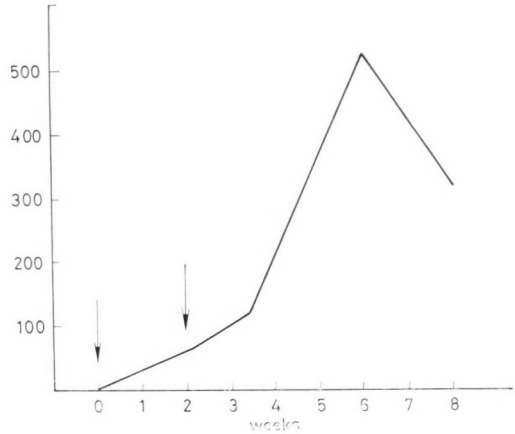

Fig. 2. Serological response after repeated administration of monovalent vaccine (geometric mean titres)

Titres of circulating antibodies during experiment No.1 are shown in Fig. 1. Significant antibody titres (1 : 64–1 : 128) developed in response to all vaccines before the 21st day after the first vaccination.

Experiment No.2 showed that revaccination on the 14th day provokes production of high titre virus-neutralizing antibodies (Fig. 2).

Experiment No.3 showed that 14 days after a single dose of bivalent vaccine 1 : 32–1 : 64 titre virus neutralizing antibodies appear against both virus types (Fig. 3).

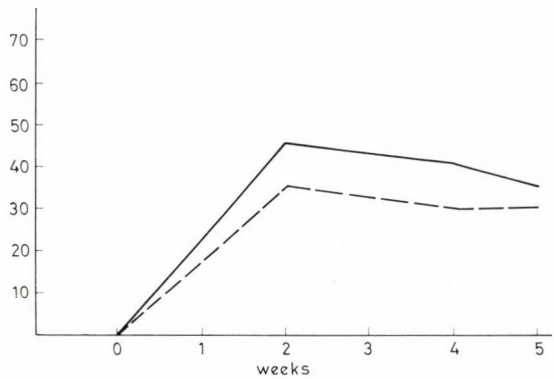

Fig. 3. Serological response after vaccination with bivalent vaccine (geometric mean titres)

Discussion

Adenovirus-induced pneumoenteritis of lambs has so far been reported only in Hungary and no data are available on the specific control of the disease. Data on vaccines against similar diseases of calves show that immunization with vaccines without adjuvant low titres (1 : 4–1 : 8) of antibodies appear [10], while TRIBE *et al.* [9] demonstrated higher titres (1 : 64–1 : 384) after administration of vaccines adsorbed in different ways. Accordingly, we developed only vaccines with adjuvants. The experiments proved that vaccines containing inactivated and adjuvated adenovirus can stimulate a proper antibody response also in sheep.

The data of experiment No.1 show that combinations of methods of inactivation and different adjuvants induced a comparable antibody response. For economy, formalin and aluminium hydroxide gel was used in the subsequent experimental vaccines.

The data of experiment No.1 also show that a second vaccination on day 21 does not increase significantly the titre of virus neutralizing antibodies. In experiment No.2, formalin inactivated and aluminium hydroxide adsorbed vaccines induced good antibody titres. Revaccination on the 14th day significantly increased the titre values. Due to the limited number of animals used in these tests, further experiments are needed to determine the optimum time for revaccination.

According to experiment No.3, after administration of a bivalent vaccine in seronegative lambs generally 1 : 32–1 : 64 titre virus neutralizing antibodies appear in 21 days. These titre values are significantly lower than those found in experiment No.1.

In field experiments, ewes with natural active immunity were vaccinated; both mono- and bivalent vaccination significantly increased their level of virus neutralizing antibodies [11]. Bivalent vaccines induced similar antibody levels as did monovalent vaccines. The serological response of seronegative fattening lambs was, however, different when they were injected with mono- and bivalent vaccines.

It has been concluded that in previously infected sheep even a small dose of antigen can stimulate a dynamic secondary antibody response. Lambs not infected before showed higher antibody levels to monovalent than to bivalent vaccines.

Summarizing the results, adenovirus vaccines induced a high level of virus neutralizing antibodies in sheep. Results of challenge tests to determine the protective value of our vaccines have been reported separately [11, 12].

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NEW SELECTIVE MEDIUM FOR THE ISOLATION OF HAEMOPHILUS SPECIES

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Chocolate agar containing lincomycin (5 μg/ml) was used for the isolation of haemophilus strains. One-hundred haemophilus strains of 3 species grew well on selective plates, whereas Gram-positive bacterial failed to form colonies on them. The selective culture proved especially advantageous when throat swabs were examined.

Haemophilus influenzae, *Haemophilus parainfluenzae* and *Haemophilus parahaemolyticus* strains are frequent pathogens of upper respiratory infections. Their aetiological role is especially significant in chronic bronchitis [1, 2]. Isolation of the pathogenic agent and determination of its antibiotic sensitivity is the basic condition of adequate treatment, but their isolation is difficult because the mixed flora of the upper respiratory tract and of the buccal cavity overgrows the haemophilus colonies. Therefore, washing of the sputum with physiological saline was recommended [1]. Vancomycin or bacitracin-containing chocolate agar facilitates the cultivation of haemophilus strains from mixed flora [3–8]. Haemophilus strains are relatively resistant to lincomycin, whereas the Gram-positive flora of the upper respiratory mucosa shows sensitivity to this antibiotic. In this way, lincomycin was found suitable for the isolation of haemophilus strains.

Materials and methods

Strains. One-hundred *Haemophilus* strains were studied; 44 were obtained from hospital patients with laryngitis, otitis, meningitis and bronchitis, and 54 from the throat swabs of healthy persons. The strains were isolated on chocolate agar.

Species determination of the strains was performed by Sim's growth factor requirement method as well as on the basis of haemolysis, sucrose and xylose decomposition, indole production and ONPG test [9]. Distribution of the 100 *Haemophilus* strains: 39 *H. influenzae*, 41 *H. parainfluenzae*, 20 *H. parahaemolyticus.*

Lincomycin sensitivity of the haemophilus strains was examined as follows. (1) *Disk method*: susceptibility paper disks (Institute for Serobacteriological Production and Research, Human, Budapest) of 10 μg lincomycin content were placed on the chocolate agar swabbed densely with the bacterial suspension in physiological saline and incubated for 24 hr. (2) *Plate dilution method*: physiological saline suspensions of the strains were seeded in sectors on chocolate agar containing 10, 5, 2.5 and 1.2 μg/ml lincomycin (lincomycin hydrochloride, USSR) and

on control chocolate agar and incubated for 24 hr. (3) *Examination of the isolated haemophilus colonies.* Of the dilutions of 5 haemophilus strains suspensed in physiological saline 0.05 ml aliquots were placed on chocolate agar containing 5 μg/ml lincomycin as well as on control plates. The number of the colonies and their morphology was compared on selective and chocolate agar plates.

Selective bacterial effect of lincomycin. The physiological saline suspensions of 220 bacteria occurring generally in the mouth and respiratory tract were seeded on chocolate agar of 5 μg/ml lincomycin content and on control chocolate agar.

Isolation of haemophilus strains from human throat mucus on selective culture media. Throat swabs taken from 50 young adults were seeded on chocolate agar of 5 μg/ml lincomycin content and on control chocolate agar. The composition of the chocolate agar was as follows. Nutrient agar was prepared from beef infusion with 2% agar and 1% Witte peptone pH 7.4. The nutrient agar was subsequently supplemented at 80 °C with 5% ox blood and the bottles were left to stand at the same temperature for 30 min.

Results

Haemophilus strains showed a relative resistance to lincomycin.

Paper disks of 10 μg licomycin content failed to show an inhibition zone with 96 strains. In the case of 2 *H. parainfluenzae* and 2 *H. parahaemolyticus* strains inhibition zones in the range of resistance were observed. As to colony morphology and microscopic picture, uninhibited growth was observed with all strains with the plate dilution method, using 5 μg/ml lincomycin concentration. This finding was confirmed by the examination of the *Haemophilus* isolates on selective and control chocolate agar.

Strains studied for selective bacterial effects are summarized in Table I.

Bacteria of the normal pharyngeal mucus belong in their majority to Gram-positive organisms and fail to form colonies even 48 hr incubation on 5 μg/ml lincomycin containing chocolate agar plates. The advantages of the selective plates were found striking when 50 throat swabs were examined. These swabs yielded a mixed flora with two exceptions where *Streptococcus mitis* was obtained in pure culture. Isolated haemophilus colonies were found on chocolate agar in 3 cultures whereas in 20 cases the haemophilus bacteria appeared in a dense mixed flora which made further isolations necessary. On selective agar plates *Neisseria pharyngis* was cultivated from 40 throats. Isolated haemophilus colonies were obtained in 33 cases, *Escherichia coli* grew from two, *Klebsiella pneumoniae* from one and *Streptococcus faecalis* from two throats. Capsulated glossy haemophilus colonies and pigmented shrinking neisseria colonies could be separated in the majority of cases by simple observation, but their microscopic picture and the intensity of the oxidase reaction showed differences. The number of haemophilus positive cases on selective plates surpassed by 20% that on the control chocolate plates. The dense mixed flora of these 10 throats covered the low number of haemophilus bacteria. In further 20 cases they were overgrown to such an extent that further isolation was required. The sensitivity of the haemophilus strains made these latter results questionable.

Table I

In vitro sensitivity of various microorganisms to lincomycin

	Number of strains	Number of strains growing on chocolate agar with lincomycin (5 μg/ml)
Streptococcus pyogenes	10	—
Streptococcus mitis	50	—
Streptococcus faecalis	20	20
Streptococcus pneumoniae	5	—
Staphylococcus aureus	10	—
Staphylococcus epidermidis	10	—
Micrococcus luteus	5	—
Aerococcus viridans	10	—
Corynebacterium diphtheriae	5	—
Corynebacterium pseudodiphtheriae	30	—
Listeria monocytogenes	1	—
Bacillus anthracis	1	—
Bacillus cereus	1	—
Neisseria pharyngis	45	45
Escherichia coli	2	2
Citrobacter freundii	1	1
Klebsiella pneumoniae	4	4
Salmonella typhi	1	1
Salmonella paratyphi-A	1	1
Shigella flexneri	1	1
Pseudomonas aeruginosa	4	4
Proteus vulgaris	1	1
Proteus mirabilis	1	1
Candida albicans	2	2

Discussion

Lincomycin is considered to be ineffective on haemophilus strains [10–14].

In earlier studies 160 cultures belonging to 3 species *(H. influenzae, H. parainfluenzae* and *H. parahaemolyticus)* were examined for their antibiotic sensitivity by the disk method. The strains were resistant to disks impregnated with 10 μg lincomycin, only 8 cultures showed a minimum inhibition zone at the limit of resistance and moderate sensitivity [15].

In the present study 100 freshly isolated haemophilus strains were subjected to the selective effect of lincomycin. A slight inhibition zone developed around the disks of 10 μg lincomycin content in 4 cultures and with the same cultures a reduced bacterial count was found on chocolate agar containing 10 μg/ml of lincomycin. Thus, having controlled the bacterial count, microscopic picture and isolated colony morphology, 5 μg/ml of lincomycin was added to the selective chocolate agar.

Lincomycin resistance characterizes the *H. influenzae* species and the unfrequent occurrence of a minimum inhibition effect was observed with the *H. parainfluenzae* and *H. parahaemolyticus* strains. These examination confirmed the strong activity of lincomycin against Gram-positive bacteria. The selective effect of lincomycin and the resistance of *Haemophilus* made the antibiotic suitable for the isolation of these bacteria from materials containing a predominantly Gram-positive flora. The results of parallel seeding on lincomycin chocolate and chocolate agar plates showed that not only the rate of positive results grew by 20% but the identification and antibiotic sensitivity determination of colonies isolated on selective plates could be carried out more simply and rapidly.

Isolation of *H. influenzae* from materials containing Gram-positive bacteria (respiratory tract, ear, eye and genital organs) is a difficult task. TURK and MAY's method of washing the sputum with physiological saline reduces with the accompanying flora the haemophilus count [1]. FÜZI described a chocolate agar with 25 μg/ml vancomycin content which gave excellent results in the isolation of *H. influenzae* [3]. KOHN recommended bacitracin which was placed in paper disks containing 100 μg of the agent on the seeded plates [4]. BABER [5] supplemented the chocolate agar with bacitracin at 10 μg/ml concentration. CRAWFORD *et al.* [6] prepared the bacitracin-containing chocolate agar with rabbit blood for distinguishing the haemolytic strains, LITTLE [7] used a combination of bacitracin with crystal violet and HANSEN *et al.* [8] with cloxacillin. Bacitracin-containing selective culture media are widely used, but the finding of EVANS [16] who isolated bacitracin sensitive haemophilus strains from human meningitis cases is noteworthy.

Chocolate agar of 5 μg/ml lincomycin content can be stored in refrigerator for 1 week without loss of effect. The medium was found effective in rapid isolation of haemophilus strains from materials contaminated with Gram-positive bacteria and provided quick and reliable assistance in the diagnosis of haemophilus infections.

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SPECIFIC ANTIBODIES TO VIRUSES HL-23 AND BILN IN THE BLOOD PLASMA OF PATIENTS WITH ACUTE MYELOGENOUS LEUKAEMIA AND WITH POTENTIAL PRELEUKAEMIA*

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Blood plasma samples from patients with acute myelogenous leukaemia (AML) or potential preleukaemia and from control subjects were tested for antibodies to the viruses HL-23 and BILN by membrane immunofluorescence. Of 15 patients with untreated AML, three, each having a low peripheral leucocyte count at the time of sampling, had detectable antibodies. Antibodies were present in the plasma of 5 out of 8 AML patients being in remission as a result of chemotherapy. In these cases, the antibody levels significantly exceeded those demonstrated in the untreated cases. Of 12 patients with potential preleukaemia, five proved to be positive. Of the 7 antibody-negative patients, four developed manifest leukaemia within 12–18 months after the first testing. The results are suggestive of a favourable prognostic role of the presence of the antibodies under study In the majority of the antibody-positive AML and potential preleukaemia cases antibodies were detectable to both components of the HL-23 virus. Of 30 control subjects, three had demonstrable antibodies to the BILN virus.

In 1975, GALLAGHER and GALLO [1] observed C-type retrovirus particles in a cell culture made from peripheral leucocytes of a patient with acute myelogenous leukaemia (AML). The virus, which was then isolated has been designated HL-23. Reisolation from the same patient during a subsequent relapse [2] proved the human origin of the virus. It was shown later that the HL-23 virus is a complex of two viruses [3], the one being related to primate leukaemia–sarcoma viruses, *viz.* simian sarcoma virus (SiSV) [4] and gibbon ape leukaemia virus (GaLV) [5], the other to an endogenous retrovirus of baboons. Of the latter, numerous strains including M7 [6] and BILN [7] have been isolated. On the surface of peripheral leucocytes from patients with AML or chronic myelogenous leukaemia (CML), METZGAR *et al.* [8] detected, by the microcytotoxicity method, an antigen related to human A204 cells infected by the HL-23 virus. Whereas the surface antigens related to SiSV and GaLV were also detectable on lymphogenous leukaemia cells, the HL-23-induced surface antigen proved specific for myelogenous leukaemias.

The above findings have prompted us to investigate the following questions.

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(i) Is there any HL-23-specific antibody detectable in the blood plasma of patients suffering from AML or a potentially preleukaemic haematological disorder, and, if so, against which component of the virus complex?

(ii) Does the presence or absence of these antibodies have any prognostic importance?

Materials and methods

Cell cultures and their maintenance. The cell cultures used originated from the following sources. The established human cell line NC-37 and the NC-37 cells infected by the BILN virus (B-1) in an infectious form were kindly supplied by Professor B. A. LAPIN (Institute for Experimental Pathology and Therapy of the Academy of Medical Sciences, Sukhumi, USSR); the A7573 cell in line developed from canine thymus cells by Dr. J. GRUBER (National Cancer Institute, Bethesda, Md, USA); the A7573 line infected by, and shedding, the HL-23 virus by Dr. R. C. GALLO (National Cancer Institute, Bethesda, Md, USA).

The cell cultures were maintained in RPMI nutrient fluid containing 10% fetal calf serum (Gibco Bio-Cult. Ltd, Paisley., Scotland). The suspended cultures were incubated at 37 °C in an atmosphere containing 5% CO_2 at 80% relative humidity (Assab Incubator, Solna, Sweden). The initial density of the cell suspension was $1–2 \times 10^5$ cells/ml. The cells were transferred when the cell count had reached 10 times the original density, i.e. at 7–10-day intervals.

Blood plasma samples. Fresh heparinized blood was centrifuged and the plasma was decanted and stored at −70 °C until used. Before performing the indirect membrane immunofluorescence test, the plasma samples were incubated at 56 °C for 30 min to inactivate the complement.

Indirect membrane immunofluorescence. The cell suspension to be used contained at least 80% viable cells. Viability was determined in 1% trypan blue solution. As target cells, A7573 cells infected by the HL-23 virus, or B-1 cells were used. The uninfected cultures, A7573 and NC-37, respectively, were used as controls.

In the first phase of the reaction 0.1 ml plasma was added to 10^6 target cells. The cells were then incubated at 37 °C for 20 min and washed three times with a PBS of pH 7.2. In the second phase, 0.05 ml of 10-fold diluted goat anti-human IgG serum labelled by FITC (Hyland, Costa Mesa, Calif., USA) was added and the cells were incubated and washed three times as above. Washed cells were dropped on a slide and allowed to dry. Then, 1 drop of glycerol–PBS (1 : 1) was added. The preparation was then covered with a coverslip and examined under the fluorescence microscope.

Results

Since the preparations containing uninfected cells showed no fluorescence, the antibody contents of the samples were expressed in percentage fluorescent cells. Figure 1 shows the fluorescing cells in a positive sample.

1. *Anti-HL-23 and anti-BILN in plasma samples from untreated AML patients.* Plasma samples from 15 untreated AML patients were tested (Table I). In the first 6 cases, the peripheral leucocyte count and among the leucocytes the per cent of blast cells were extremely high. In the remaining 9 cases the leucocyte count was subnormal or at most twice the normal value and the number of blast cells did not exceed 50% of the leucocytes. Antibodies could be demonstrated only in this second group, in plasma samples of three patients. In two of the positive cases the anti-HL-23 level was higher than the anti-BILN level, in the third case the two levels were equally high.

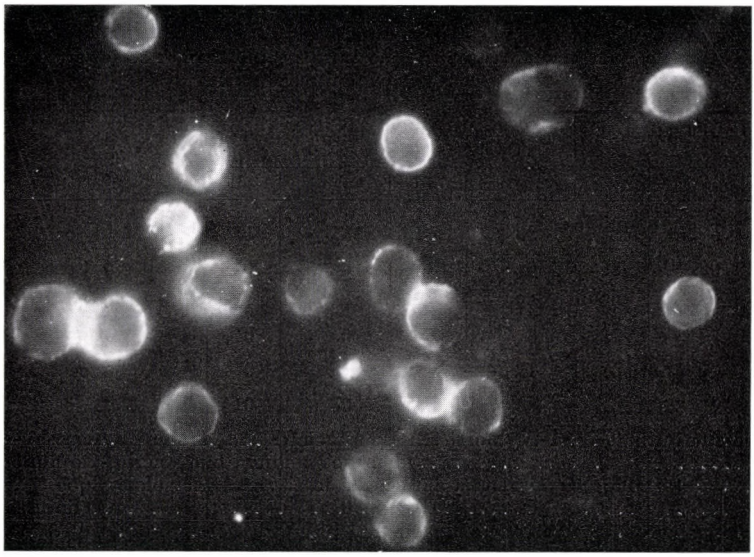

Fig. 1. Indirect membrane immunofluorescence with a plasma sample from N. A., a 63-year-old preleukaemic patient with sideroblastic anaemia. Target cells A7573 carrying the HL-23 virus

Table I

Anti-HL-23 and anti-BILN antibodies in the blood plasma of patients with untreated AML

Patient			Diagnosis	Leucocyte		Antibody level*	
Name	Sex	Age, years		per μl	blast per cent	anti-HL-23	anti-BILN
K. I.	♂	54	AML	150 000	86	0	0
N. I.	♂	54	AMMoL	174 000	91	0	0
T. T.	♂	43	AMMoL	158 000	96	0	0
P. I.	♂	32	AMMoL	536 000	95	0	0
M. P.	♀	62	AML	28 500	93	0	0
K. Gy.	♂	44	AML	45 000	97	0	0
K. I.	♂	44	AML	4 500	50	0	0
E. K.	♂	75	AML	1 900	14	0	0
J. K.	♂	50	AML	5 200	28	0	0
Sz. J.	♂	49	AML	1 600	15	0	0
B. G.	♂	69	AML	4 600	24	0	0
V. P.	♂	65	AMMoL	11 600	36	0	0
B. I.	♂	47	AML	2 400	8	20	20
Gy. I.	♀	70	AMMoL	13 600	16	30	10
P. S.	♀	43	AMMoL	13 000	51	40	15

Abbreviations: AML = acute myelogenous leukaemia
AMMoL = acute myelomonocytic leukaemia
* Expressed in fluorescing cells, per cent

2. *Dynamics of anti-HL-23 and anti-BILN levels in the plasma of AML patients.* Plasma samples from four patients with AML and four with acute myelomonocytic leukaemia (AMMoL) were examined during chemotherapy. Lasting remission was observed in the first five patients (Table II). All samples taken during the acute phase of the disease proved negative, and the antibodies appeared exclusively during remission. In two of the five antibody-positive cases the anti-HL-23 level was higher than the anti-BILN level, one patient had the two antibodies at the same level and in the remaining two cases antibodies could be detected with HL-23-infected target cells but not with the BILN-infected ones.

Table II

Dynamics of the anti-HL-23 and anti-BILN levels in the plasma of patients with AML

Patient			Diagnosis	Antibody levels* during			
				progression		remission	
Name	Sex	Age, years		anti-HL-23	anti-BILN	anti-HL-23	anti-BILN
N. I.	♂	44	AMMoL	*0*	0	80	20
K. Gy.	♂	44	AML	0	0	40	0
Gy. I.	♀	70	AMMoL	0	0	70	5
V. P.	♂	65	AMMoL	0	0	40	0
B. I.	♂	47	AML	0	0	60	60
B. G.	♂	69	AML	0	0	0	0
K. I.	♂	54	AML	0	0	0	0
K. F.	♂	30	AMMoL	0	0	0	0

Abbreviations: AML = acute myelogenous leukaemia
AMMoL = acute myelomonocytic leukaemia
* Expressed in fluorescing cells, per cent

3. *Anti-HL-23 and anti-BILN in plasma samples from potentially preleukaemic patients.* The results for the 12 potentially preleukaemic patients are summarized in Table III. We succeeded in detecting antibodies in 5 cases. In four of these the anti-HL-23 level was higher than the anti-BILN level, in the remaining case there was no difference between the two levels. Four of the seven antibody-negative cases had developed manifest acute leukaemia 12–18 months after the beginning of our studies.

In the control group including 30 subjects, partly healthy ones, partly patients suffering from non-tumorous disease, five had cytopenia which was qualified as non-preleukaemic. Antibodies to the BILN virus were detected in three cases, at very low levels (10–15% of the cells were fluorescing). None of the antibody-positive samples originated from patients with cytopenia. Antibodies to the HL-23 virus could be detected in none of the 30 samples.

Table III

Anti-HL-23 and anti-BILN antibodies in the plasma of patients with preleukaemia

Patient			Diagnosis	Antibody levels*		Appearance of leukaemia
Name	Sex	Age, years		anti-HL-23	anti-BILN	
N. A.	♀	63	sideroblastic anaemia	80	30	—
I. I.	♂	22	aplastic anaemia	60	30	—
P. F.	♂	30	aplastic anaemia	40	40	—
N. P.	♀	45	sideroblastic anaemia	30	10	—
M. I.	♂	66	pancytopenia	80	50	—
T. J.	♀	69	sideroblastic anaemia	0	0	—
F. K.	♀	22	haemolytic anaemia	0	0	—
K. Gy.	♂	47	thrombocytopenia	0	0	—
P. J.	♂	47	haemolytic anaemia	0	0	AML
P. M.	♂	25	refractory anaemia	0	0	AEL
Sz. M.	♀	14	pancytopenia	0	0	ALL
F. L.	♂	60	pancytopenia	0	0	ALL

Abbreviations: AML = acute myelogenous leukaemia
AEL = acute erythroid leukaemia
ALL = acute lymphoid leukaemia
* Expressed in fluoresceing cells, per cent

Discussion

The present results indicate that antibodies to C-type primate retroviruses may be present in the blood plasma of patients with AML or potential preleukaemia and also in control subjects. There was, however, a difference between these groups in the specificity and in the plasma level of the antibodies. In the plasma samples taken from control subjects only antibodies binding to the surface antigen(s) of the endogenous baboon virus could be detected.

It is well known that the genome of healthy human cells contains a virogene [9] related to the genome of the baboon endogenous virus [9]; this gene may be expressed even without malignant transformation [10]. In contrast, nucleic acid sequences related to those of primate leukaemia–sarcoma viruses could be detected in malignant tumorous cases only. The difference was demonstrated when nucleic acids of human AML cells were subjected to hybridization experiments [11]. This has been indirectly confirmed by our results, *viz.* antibodies specific for the oncogene component of the HL-23 virus could not be detected in plasma samples collected from control subjects. In cases where the plasma level of anti-HL-23 was higher than that of anti-BILN, the difference has been attributed to the presence of antibodies against surface antigens induced by the oncogenic virus component. As demonstrated in nu-

cleic acid hybridization experiments [12], the expression of nucleic acid sequences of endogenous baboon C-type virus was more pronounced in leucocytes of AML patients than in those of control subjects. It is therefore not surprising that anti-BILN was detected in plasma samples collected from patients with AML and those with potential preleukaemia more frequently than in control samples.

In only 3 of the 15 untreated cases of AML were antibodies demonstrable; the peripheral leucocyte count was low at the time of sampling in each of the positive cases. From the cases under chemotherapy, antibodies to virus-induced surface antigens could not be detected except in remission, and the antibodies found in this phase considerably exceeded in level those observable sometimes in untreated cases. These results seem to be suggestive of a favourable prognostic value of the detection of these antibodies. Since the target cells used in the demonstration of antibodies, though supporting the reproduction of the respective virus, are not transformed by the virus, the present conclusions are valid for the human response to virion components appearing on the cell surface, *viz.* p12, p15 (E), gp45 and gp70 antigens [13–16].

Since these antigens are present both in the virion envelope and on the surface of the transformed cells, the antibodies produced against them are both virus-neutralizing and cytotoxic in effect. This has been proved by experiments performed on gibbons infected by GaLV [17].

The prognostically favourable role of the presence of the antibodies under study seems to be supported, first of all, by the results of testing of patients with potential preleukaemia. The relationship between the expression of the retrovirus genome and the development of a preleukaemia into manifest leukaemia seems to be supported by results obtained during investigations into reverse transcriptase [18], detection of C-type particle by electron microscopy [19] and detection of p30 antigens [20].

Among the 12 preleukaemia patients, 5 had plasma antibody to the HL-23 virus and none of the four who developed manifest leukaemia had had antibodies at the first testing. It is therefore reasonable to assume that the antibody response to antigens of the virus-carrying cell and to virion surface antigens may be factors inhibiting the cell proliferation induced by retrovirus expression.

Reliable judgement of the prognostic value of HL-23 virus-specific antibodies requires investigation of a larger number of AML patients and patients with potential preleukaemia and, further, attempts to demonstrate these antibodies in the environments of patients.

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INFLUENCE OF AMYLASE EXCRETION ON BIOMASS PRODUCTION BY AMYLOLYTIC YEASTS

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A survey of some amylolytic yeasts has been made with the view to produce single cell protein (SCP) from starchy substrates. On yeast nitrogen base (YNB, Difco) media, the pH quickly falls to below 3.0, amylases remain intracellular and biomass yields are low. On YNB buffered media α-amylases are excreted and yields are high, but the growth rates do not improve. The influence of pH and amylase excretion on biomass yield and growth rates are important for SCP production.

Single cell protein production from yeasts has steadily developed over a number of years. A survey by MOO-YOUNG [1] shows that 42 out of 54 SCP producers in the world use yeasts. Starch stands out among the carbon substrates of potential because it is cheap, non-toxic and renewable [2]. Yeast culture on starch demands a first stage of hydrolysis. A number of processes have been put forward to accomplish this for the treatment of waste water from potato industries. These include hydrolysis by either chemical [3, 4] or enzymatic means [5, 6]. The Symba process [7–9] uses two yeasts in association: *Endomycopsis fibuligera* assures enzyme production for hydrolysis whereas *Candida utilis* grows rapidly on the hydrolytic products. In most other cases, *C. utilis* is the important food yeast. It should be interesting to obtain only one strain capable of performing both functions of hydrolysis and biomass production. The use of *Endomycopsis fibuligera* can be ruled out due to its slow rate of growth on starch. Some research workers are using the technique of protoplast fusion with the hope of obtaining a strain with the combined properties of amylolysis of *E. fibuligera* and the growth characteristics of *C. utilis* [10].

A survey of a large number of yeasts has been made with the view to finding a yeast strain capable of simultaneously assuring rapid starch hydrolysis and growth.

Materials and methods

Biological materials. The following yeast strains from the Centraalbureau voor Schimmelcultures (Delft, Netherland) were tested: *Candida blankii* Bruckey et van Uden CBS 1898, *Candida bogoriensis* Deinema CBS 4101, *Candida brumptii* Langeron et Guerra CBS 6145, *Candida cifferii* Kreger van Rij CBS 5646, *Candida glaebosa* Komagata et Nakase CBS 5691,

Candida humicola (Daszewska) Diddens et Lodder CBS 2822, *Candida langeronii* Dietrichson, ex van Uden et Buckley CBS 227, *Candida mogii* Vidal-Leiria CBS 2032, *Candida oregonensis* Phaff et Docarmo-Sousa CBS 5623, *Candida sake* (Saito et Ota) van Uden et Buckley nov. comb. CBS 5690, *Candida viswanathii* Sandhu et Randhavia CBS 2784, *Cryptococcus flavus* (Saito) Phaff et Fell nov. comb. CBS 331, *Cryptococcus laurentii* var. *flavescens* (Kufferath) Skinner CBS 5539, *Cryptococcus* var. *flavescens* Saito, Lodder et Kreger van Rij, *Cryptococcus infirmo-miniata* (Okunuki) Phaff et Fell nov. comb. CBS 323, *Debaryomyces marama* Di Menna CBS 1958, *Debaryomyces phaffii* Capriotti CBS 4346, *Debaryomyces vanriji* (van der Walt) et (Tscheuschner) Abadie, Pignal et Jacob CBS 3024, *Endomycopsis capsularis* (Schionning) Dekker CBS 2519, *Endomycopsis fibuligera* (Lindner) Dekker CBS 6310, *Hansenula capsulata* Wickerham CBS 1993, *Hansenula cifferii* Lodder CBS 1990, *Hansenula fabianii* Wickerham CBS 5640, *Hansenula holstii* Wickerham CBS 4140, *Lipomyces lipofer* Lodder et Kreger van Rij CBS 944, *Lipomyces kononenkoae* Nieuwdorp, Bos et Sloof CBS 2514, *Lipomyces starkeyi* Lodder et Kreger van Rij CBS 1807, *Pichia burtonii* Boidin CBS 6141, *Pichia media*, Boidin, Pignal, Lehodey, Vey et Abadie CBS 5521, *Pichia vini* (Zimmerman) Phaff var. *vini* CBS 4328, *Rhodotorula marina* Phaff, Mrak et Williams CBS 4516, *Schwanniomyces alluvius* Phaff, Miller et Cooke CBS 4515, *Schwanniomyces castellii* Capriotti CBS 2863, *Schwanniomyces occidentalis* Klocker CBS 1153, *Schwanniomyces personii* van der Walt CBS 4869, *Torulopsis colliculosa* (Hartmann) Saccardo CBS 133, *Torulopsis ernobii* Lodder et Kreger van Rij CBS 1737, *Torulopsis ingeniosa* Di Menna CBS 4240, *Wingea robertsii* van der Walt CBS 4639.

Conditions of culture. Cultures were made in 6 litre Erlenmeyer flasks filled to a tenth of their volume and shaken at 28 °C. Culture media were as follows. *YE medium:* yeast extract 0.2%, soluble starch (Prolabo) 0.4%. *YNB glucose medium:* Yeast nitrogen base (Difco) (YNB) 0.67%, glucose 0.4%. *YNB starch medium:* yeast nitrogen base (Difco) 0.67%, starch 0.4%. *YNB medium pH 3.5:* yeast nitrogen base (Difco) 0.67%, 0.05 M sodium diphosphate ($Na_2HPO_4 \cdot 12H_2O$), 0.035 M tartaric acid solution containing 0.4% starch. *YNB medium pH 5.5:* yeast nitrogen base (Difco) 0.67%, 0.05 M sodium diphosphate ($Na_2HPO_4 \cdot 12H_2O$), 0.025 M tartaric acid solution containing 0.4% starch. *YNB medium pH 7.0:* yeast nitrogen base (Difco) 0.67%, 0.4% starch dissolved in 0.02 M sodium phosphate buffer.

The neperian growth rates were determined from experimental growth curves obtained by following the optical densities of culture media by means of a Klett–Summerson photoelectric colorimeter at 420 nm. Growth rates (μ) were calculated from the equation $\mu = \frac{0.693}{T}$ where T = generation time determined graphically. Amylase activities were determined on culture filtrate and broken cells by a measure of iodine decoloration according to the SMITH and ROË method [11].

Results

The growth rates of all the yeast strains were measured in YNB starch medium. Eleven strains had generation times less than 5 hr. A large number of those remaining had generation times above 8 hr under our experimental conditions. The former were selected for further studies.

1. *Study of the potentials of the yeasts.* The 11 strains selected were cultivated on YNB glucose, YNB starch and YE starch media. Growth rates were higher on glucose than on starch (Table I). On YE starch medium, certain strains showed fast growth rates, the sensibility approached the values observed on glucose: *C. oregonensis*, *P. burtonii*, *T. ingeniosa* and *S. occidentalis*.

The biomass obtained (dry weight/litre) was always less on YNB starch than on YNB glucose. The starch was never completely degraded; it is probable that the cell wall amylases can attack only the side chains of the starch molecules and are prevented from reaching the "nucleus" because of steric hindrance. Biomass yield is, however, calculated with reference to initial amounts

Table I

Growth rate (μ per hour), dry weight (g/litre) and biomass yield (%) of 11 yeasts on different non buffered media

	YNB glucose medium			YNB starch medium			Yeast extract starch medium	
	Growth rate, μ/hr	Dry weight, g/l	Yield, per cent	Growth rate, μ/hr	Dry weight, g/l	Yield, per cent	Growth rate, μ/hr	Dry weight, g/l
E. fibuligera	0.41	1.44	36	0.19	1.24	31	0.32	1.88
S. alluvius	0.35	1.60	40	0.16	0.46	14	0.28	2.68
S. castellii	0.46	1.88	47	0.19	1.04	26	0.30	2.32
S. occidentalis	0.38	1.04	46	0.19	0.52	13	0.35	2.44
H. capsulata	0.41	1.64	41	0.17	1.12	28	0.32	2.44
P. vinii	0.38	1.88	47	0.20	1.12	28	0.30	2.52
C. oregonensis	0.41	1.84	46	0.17	0.96	24	0.41	2.60
C. brumptii	0.41	1.44	36	0.17	0.64	16	0.35	2.20
L. starkeyi	0.41	1.24	31	0.17	0.92	23	0.35	1.60
P. burtonii	0.41	1.76	44	0.17	1.24	31	0.39	2.36
T. ingeniosa	0.41	1.80	45	0.17	1.28	32	0.38	2.40

of starch. On YE starch medium, the starch is almost completely degraded, due to the excretion of large quantities of amylases into the culture medium. Biomass yield is thus important. Here it is impossible to calculate yield because the constituents of yeast extract can itself assure a certain amount of growth. In addition, it appears that enzyme excretion is beneficial to total growth. Finally, the YE starch culture was always around pH 5.0. On the other hand, that of the YNB starch medium always fell to pH 3.0. It is not impossible that such acidification halts growth entirely thus resulting in an underestimation of the yield.

In order to study the respective influence of the pH of the culture medium and of enzyme excretion, the growth rates and biomass yield of the 11 strains were studied on 3 media, buffered to different pH levels.

2. *Study on different buffered media.* The 3 buffered culture media were YNB starch medium pH 3.5, YNB starch medium pH 5.5, YNB starch medium pH 7.0 described in Materials and methods. The results are presented in Table II.

In all cases about 80% of amylase activity is released into the culture medium.

At pH 3.5, some amount of starch always remained in the culture broth at the end of culture, resulting in a low biomass yield. In all cases, the yield was higher than that observed on YNB starch medium.

Table II

Growth rate, dry weight and biomass yield of 11 yeasts on YNB starch buffered media

	YNB starch medium pH 3.5				YNB starch medium pH 5.5				YNB starch medium pH 7.0			
	Final pH	Growth rate, μ/hr	Dry weight, g/l	Yield, per cent	Final pH	Growth rate, μ/hr	Dry weight, g/l	Yield, per cent	Final pH	Growth rate, μ/hr	Dry weight, g/l	Yield, per cent
E. fibuligera	2.8	0.12	1.12	28	4.3	0.12	1.56	39	5.6	0.11	1.20	30
S. alluvius	3.0	0.17	0.76	19	4.3	0.17	1.60	40	5.6	0.23	1.32	33
S. castellii	3.1	0.19	0.96	24	4.3	0.14	1.40	35	5.6	0.08	1.28	32
S. occidentalis	3.0	0.13	0.92	23	4.3	0.18	1.60	40	5.6	0.18	1.52	38
H. capsulata	3.0	0.14	1.08	27	4.5	0.13	1.52	48	5.5	0.12	1.68	42
P. vinii	2.9	0.21	1.20	30	4.7	0.14	1.52	38	5.4	0.09	1.32	33
C. oregonensis	3.0	—	0.32	8	4.5	0.14	1.64	41	5.8	0.09	1.20	30
C. brumptii	3.0	0.15	1.08	27	4.6	0.14	1.92	48	5.3	0.10	1.60	40
L. starkeyi	3.1	—	0.40	10	4.3	0.12	1.60	40	6.6	0.23	0.28	7
P. burtonii	3.0	0.19	0.96	24	4.5	0.17	1.40	35	5.3	0.23	1.20	30
T. ingeniosa	3.1	0.12	1.36	34	4.4	0.14	1.64	41	5.8	0.08	1.64	41

At pH 5.5, practically all the starch is hydrolysed; only some dextrins remain. Yield values here are comparable to those found on glucose.

At pH 7.0, the yield is always inferior to that obtained at pH 5.5, and starch hydrolysis also improved.

It therefore appears that the addition of these different buffers provokes amylase excretion in all the yeast strains, at all pH values. The mechanism of this phenomenon is not known. In all cases, enzyme excretion leads to better starch hydrolysis and increases in biomass yield. The pH of the culture medium plays a significant role on growth rates and biomass yield. The optimum pH is not necessarily the same for all the yeast strains; this must depend to some extent on the optimum pH of the amylase of each strain.

Discussion

Yeast amylases have been tagged exocellular [12] and endocellular [13]. The results presented here show that all these amylases show an identical behaviour; the enzyme remains in the cell wall or can be excreted depending on the composition of the culture medium. Enzyme excretion does not seem to depend on the pH. The mineral salt content of the medium plays an important role in the mechanism of excretion. This will be the subject of a next study.

Yeasts are not capable of hydrolysing starch completely whenever the amylases are mural. On the contrary, excretion ameliorates hydrolysis and ensures total starch degradation. Evidently, it is necessary to know the optimum pH and the limiting pH of each amylase. The optimum pH of the amylase and growth of the yeast may not necessarily be the same; *L. starkeyi* for example has an optimum pH of growth at 5.5 [13], whereas its amylase has an optimum pH of 3.5. Growth of *E. fibuligera* on the other hand, is little affected by the pH. The values of biomass yield (0.35–0.48) observed on YNB starch medium pH 5.5 are comparable to the value (0.43) obtained by the Symba process [14]. In addition, the growth rates observed are higher than 1.10, the working growth rate used in the Symba process.

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DIFFERENCE IN THE VIRULENCE OF UREAPLASMA UREALYTICUM ISOLATES

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The virulence of *Ureaplasma urealyticum* strains was tested by intrarenally infecting white mice of undefined, LATI (Gödöllő, Hungary) and BALB/c breed. Freshly isolated human strains differed in virulence; when subcultured in U-9 medium they showed a gradual loss of virulence. Ureaplasma strains of calf and swine origin were virulent to mice in a medium degree. Some of the inoculated mice showed the signs of latent infection.

Shepard in 1954 described the *Ureaplasma urealyticum* strains under the name T mycoplasma. Since then, much work has been done to estimate the features of these strains, to determine their role in human diseases and also to serotype the ureaplasma isolates. It was found that the *U. urealyticum* strains of human origin are antigenically distinct from all other recognized species of *Mycoplasmatales* [1]. Till 1978, eight distinct serotypes were reported [2]. The pathogenic role of the *U. urealyticum* strains of human origin was demonstrated in urethritis by intraurethral inoculation of *U. urealyticum* organisms [3].

The pathogenic role of ureaplasmas has been suggested in human sterility [4–6], in puerperal sepsis and pelvic inflammation [7–9], in pyelonephritis and in the formation of renal calculi [10–12]. There was no clear-cut correlation between the serotype and virulence of *U. urealyticum* but in urethritis serotype 4 was the most frequent [2].

Our hypothesis was that there must be some differences between the virulence of the *U. urealyticum* strains without any correlation to the serotype. Other research workers, probably on the basis of the same hypothesis, attempted to work out virulence tests in laboratory animals, without, however, much success [13].

Materials and methods

Modified A-5 medium [14] was applied for the isolation of *U. urealyticum* strains. Trypticase soy broth powder (BBL 11 768), 4.8 g was dissolved in 163 ml distilled water, the pH adjusted to 5.5 with 2 N HCl, and Ionagar No. 2 (Oxoid 076 1527), 1.73 g was added and sterilized by autoclaving at 121 °C for 15 min then cooled to 56 °C in a water bath and converted to complete medium by the addition of 40 ml sterile horse serum previously heated to 56 °C, 2 ml of sterile yeast extract and potassium penicillin G, 200 000 IU.

Preparation of yeast extract according to PRATT [15] : 1000 g of fresh baker's yeast is suspended in 500 ml distilled water with continuous stirring. The suspension is heated to 80 °C. The pH is adjusted with concentrated HCl to 4.5 and the suspension is incubated 20 minutes at 80 °C. After sedimentation at room temperature the supernatant is decanted, centrifuged, and filtered through G-4 glass filter (Carl Zeiss, Jena). The pH of the filtrate is adjusted with N NaOH to 7.4 and filtered through G-5 glass filter (Carl Zeiss, Jena). The sterile yeast extract is stable for months at −20 °C.

U-9 medium [16]. Tryptic digest broth (Difco 0977-01) 0.75 g; NaCl, 0.5 g; KH_2PO_4, 0.02 g; dissolved in 100 ml distilled water. The basal medium is sterilized by autoclaving at 121 °C for 15 min. The basal medium is completed by adding to 95 ml amount: unheated, filter sterilized horse serum, 4 ml; filter sterilized 10% urea stock-solution, 0.5 ml; 1% autoclave-sterilized sodium phenol red solution, 0.1 ml; sterile potassium penicillin G from a 100 000 IU/ml stock solution, 1 ml. The final reaction of the medium should be pH 6 ± 0.2. The complete medium was aseptically dispensed in 2 ml portions to Widal tubes. The dispensed medium can be stored for 1 month at 4 °C.

LSB medium [17] was used for culturing *U. urealyticum* strains of animal origin. LSB basal medium: tryptic digest broth, 100 ml; 1% NaCl, 100 ml; dextrose, 0.2 g; sterilized by autoclaving at 115 °C for 25 min. The basal medium is completed by adding sterile unheated horse serum, 58 ml; 0.2% yeast extract (Oxoid), 29 ml; filter-sterilized 10% urea stock solution, 0.84 ml; 1% autoclave-sterilized sodium phenol red solution, 0.6 ml; sterile potassium penicillin G from a 100 000 U/ml stock-solution, 1.7 ml. The reaction of the medium should be about pH 6.

Vaginal, cervical and urethral mucus was taken by a loop, and the media were directly inoculated, incubated at 37 °C and examined daily. Ureaplasma growth was revealed on solid A-5 medium by an indicator solution: equal quantities of 10% urea solution and 0.8% $MnSO_4$ solution were mixed and 1 ml was pipetted to each solid medium, and after 1 minute the solution was poured off. In this way the *U. urealyticum* colonies turned black on account of the black colour of the reduced manganese. In U-9 media the red colour of phenol red indicates the alkaline pH, i.e. the growth of ureaplasmas. If the slightest turbidity could be observed, the absence of bacterial growth was checked by hanging drop examination and inoculation of culture media.

Inoculation of mice. U-9 media (prepared without urea) were inoculated with freshly isolated *U. urealyticum* strains and incubated at 37 °C for 18 hr. After a longitudinal cut through the abdomen, 0.1 ml of the culture was injected into the left kidney of the mouse. The abdomen was sewn together and the mice were killed at different intervals after the inoculation. The organs or organ pieces, taken aseptically from the mice were placed into U-9 media. The characteristic colour change (urealytic activity) indicated ureaplasma growth. The growth was then confirmed by inoculation into A-5 solid media.

In the case of ureaplasma strains of animal origin, LSB medium was used instead of U-9, because of the poor growth of these strains in U-9 media.

Twenty mice each were inoculated with an *U. urealyticum* strain and on every occasion 5 mice were killed.

The ureaplasma culturing results of the organs shown in the Tables are averages. Differences occurred sometimes between the culturing results of the five mice. There were some from which ureaplasma could not be reisolated or only from certain organs.

Results

The present study made on male and female patients with various genital tract disorders and asymptomatic carriers (controls) showed 60–70% ureaplasma infection of the urogenital tract (Table I).

As can be seen from Table II, of the freshly isolated *U. urealyticum* strains of human origin inoculated into mice, some could not be reisolated after five days, not even from the infected left kidney. Some other strains could be reisolated after five days only from the infected kidney, but not after ten days. In the case of two *U. urealyticum* strains the reisolation attempts were successful not only from the infected kidney, but from the non-infected kidney and from the urinary bladder even after ten days.

Table I

Frequency of U. urealyticum isolated from the urethral and vaginal mucus of adult patients with various genitourinary tract disorders and from asymptomatic pregnants in the first trimester

	Number of examined persons	Number of *U. urealyticum* positive persons	Ureaplasma positivity, per cent
Patients with genitourinary tract disorders	561	350	62
Asymptomatic pregnants	74	52	70

Table II

Survival and growth of U. urealyticum strains isolated from patients with various genitourinary tract disorders in white mice of undefined breed

Strains	Ureaplasma positivity after 5 days			Ureaplasma positivity after 10 days		
	not inoculated kidney	inoculated kidney	bladder	not inoculated kidney	inoculated kidney	bladder
3	—	—	—	—	—	—
2, 4, 5, 9, 10	—	+	—	—	—	—
7	—	—	+	—	—	—
8	—	+	+	—	—	—
1, 6	+	+	+	+	+	+

To obtain reliable results, after the first series made in mixed white mice we used BALB/c and LATI mice. To compare their sensitivity to ureaplasma, the two mouse strains were inoculated parallel with three ureaplasma strains. The results are seen in Table III; BALB/c and LATI mice behaved similarly.

To find a correlation between the multiplication and surviving capacity of the ureaplasma strains inoculated into the mouse kidney and their human pathogenicity, the mouse "virulence" of the ureaplasma strain was compared in 17 cases with the presumed human pathogenicity of the same strain. As can be seen from Table IV, the mouse "virulent" ureaplasma strains, i.e. those that could be reisolated after 10 days from the infected mice, were isolated from patients suffering from urethritis, prostatitis, kolpitis or sterility where with one exception the ureaplasma seemed to be the only pathogenic agent. Only in a single case was the "mouse virulent" ureaplasma associated with

Table III

Survival and growth of U. urealyticum in BALB/c and LATI mice

Strains	Days after inoculation	Inoculated kidney		Not inoculated kidney		Bladder		Uterus		Testicle	
		BALB/c	LATI	BALB/c	LATI	BALB/c	LATI	BALB/c	LATI	BALB/c	LATI
15	4	−	+	−	−	−	+	−	−	−	−
	7	+	+	−	+	−	+	−	+	−	+
	10	+	+	−	−	+	+	−	−	−	−
16	5	+	+	−	−	−	−	nd	nd	−	−
	10	−	−	−	−	−	−	nd	nd	−	−
	17	−	−	−	−	−	−	nd	nd	−	−
19	5	+	+	−	−	+	+	+	+	nd	nd
	11	+	+	−	−	+	+	+	+	nd	nd

+ Ureaplasma growth from the organs in U-9 medium; growth was proved by subculturing on A-5 media
nd = not done

thrush. Unfortunately the investigation was incomplete, since there was no possibility to demonstrate *Chlamydia trachomatis* (genitalis), one of the common agents causing genitourinary tract disorders besides *Trichomonas vaginalis*, *Neisseria gonorrhoeae* and *Candida albicans*.

Because of the inconsistent results of the repeated experiments with the same ureaplasma strain, we examined the "mouse virulence" after serial passage on U-9 medium (Table V). Gradual loss of the mouse virulence was observed during serial subculturing in U-9 medium of *U. urealyticum* strain Ny-13. On the other hand, strain Ny-13 had an equally good growth in U-9 medium during passages. Repeated experiments with a few other ureaplasma strains gave the same results.

The phenomenon of "latent" ureaplasma infection could be observed on two occasions in the mouse inoculation experiments, when the ureaplasma could be isolated only from a few of the infected mice and only from one single organ (Table VI).

To compare them with strains of human origin, three *U. urealyticum* strains of animal origin were examined. These strains were isolated from chicken, calf and swine and were subcultured in LSB medium. These ureaplasma strains were reported [17] to differ in serotype from those of the human strains. The strains of animal origin were inoculated into BALB/c mice kidneys. The culture medium was LSB because of the insufficient growth of these strains in U-9 medium (Table VII). As can be seen, swine and calf ureaplasmas had a medium "virulence" in mice, but the chicken strain was avirulent.

Table IV

"Mouse virulence" of U. urealyticum strains from patients with various genitourinary tract disorders

Strain	Diagnosis	*U. urealyticum* recovered from BALB/c mice		Presence of other pathogenic agents		
		4–6 days postinoculation	8–10 days postinoculation	1	2	3
11, male	Urethritis, acute	+	–	–	–	–
12, male	Urethritis, chronic prostatitis	+	+	–	–	–
13, male	Urethritis, acute	+	+	–	–	–
14, male	Gonorrhoea	+	–	+	–	–
15, female	Kolpitis	+	+	–	–	–
16, female	Kolpitis	+	–	–	–	–
17, female	Kolpitis	–	–	–	+	–
18, female	Cystitis, fluor	–	–	–	–	–
19, female	Kolpitis	+	+	–	–	+
20, male	Gonorrhoea	+	–	+	–	–
21, female	Control	–	–	–	–	–
22, female	Control	–	–	–	–	–
23, male	Gonorrhoea	+	–	+	–	–
24, male	Gonorrhoea	–	–	+	–	–
25, male	Gonorrhoea	+	–	+	–	–
26, male	Sterility	+	+	–	–	–
27, female	Sterility	+	+	–	–	–

1 = *Neisseria gonorrhoeae*
2 = *Trichomonas vaginalis*
3 = Yeasts

Table V

"Virulence" of U. urealyticum strains in BALB/c mice during subculturing in U-9 medium

Number of subcultures	Days after mouse inoculation	Inoculated kidney	Not inoculated kidney	Bladder	Uterus
1	2	+	–	–	–
	4	+	–	–	+
	6	+	+	+	+
	10	+	–	+	+
16	4	+	+	+	+
	10	–	–	–	–
31	4	–	–	–	–
	10	–	–	–	–

Table VI

Latent U. urealyticum infection in BALB/c mice

Strain	Days after inoculation	Inoculated kidney	Not inoculated kidney	Bladder	Uterus
Ny 21	2	—	—	—	—
	4	—	—	—	—
	6	—	+ (1 of 5)	—	—
	10	—	—	—	—
Ny 25	2	—	—	—	—
	5	—	+ (1 of 5)	—	—
	9	—	—	—	—
	10	+ (1 of 5)	—	—	—

Table VII

"Virulence" of U. urealyticum strains of calf, swine and chicken origin in BALB/c mice

Strain	Days after inoculation	Inoculated kidney	Not inoculated kidney	Bladder	Uterus	Testicles
Calf 1158	2	+	+	+	+	—
	6	+	—	+	+	—
	14	+	—	+	—	—
Swine 917	2	+	—	+	—	—
	6	+	—	+	—	—
	14	+	—	+	—	—
Chicken T 916	2	+	—	+	—	—
	6	+	—	+	—	—
	14	—	—	—	—	—

Discussion

The genitourinary tract of the sexually active adult population is infected with *U. urealyticum* strains in a high percentage. Different authors found 11 to 100% of infection among asymptomatic individuals [18, 19]. We found an incidence of 70% among pregnants in the first trimester, while in virgins and adolescents the infection rate is only 0 or a few per cent [12, 19–21].

A recent study has demonstrated the aetiological role of *U. urealyticum* in urethritis by the intraurethral inoculation of live *U. urealyticum* organisms

in human volunteers [3]. An aetiological role in some other conditions has also been suggested. It is supposed that there are differences in virulence between *U. urealyticum* isolates of human origin. A group of authors tried to find a correlation between the serotype and the pathogenicity of *U. urealyticum* strains. In a few cases it has been demonstrated that in some conditions most of the isolated strains belonged to one serotype. *U. urealyticum* type 4 was associated with non-gonorrhoeal urethritis in 67%, with non-bacterial pyuria in 50%, and non-bacterial chronic prostatitis in 31%, while renal stone patients had type 1 in 30%. In spite of these results the correlation between serotype and the aetiologic role of *U. urealyticum* isolates is not certain [2].

Others [13] tried to find an animal model to examine the pathogenic role of ureaplasmas of human origin. The mouse mammary gland was found suitable for *U. urealyticum* propagation. From the number of colony forming units in the gland homogenate, conclusions could be drawn as to the "virulence" of the *U. urealyticum* strain. The method is laboursome and presumably this is why it was not applied in a greater number of experiments.

In the first part of our experiments 50 undefined, later 50 each of LATI (Gödöllő, Hungary) and BALB/c white mice were killed and different organs of the mice were cultured for *U. urealyticum*. On the employed A-5 and U-9 media we could not find a single growing ureaplasma strain. So the natural infection of our mice with *U. urealyticum* strains could be excluded. Exclusion of a natural infection was necessary because *U. urealyticum* strains were isolated from numerous animal species such as monkeys, cattle, sheep, goats, dogs, swine, chicken, turkeys [17, 22–25].

Our results showed a significant difference between *U. urealyticum* strains in their growth and survival in mice organs. Numerous strains could not be isolated from the infected mice after two days and there were strains which could be isolated even after 3 weeks; 5 and 10 days were considered to be the distinctive level.

From the infected kidney the "virulent" ureaplasma strains often infected the urinary bladder, the uninoculated kidney, the testicles and also the uterus. We never found an infected embryo in the pregnant mice. After three weeks, all of the infected mice became negative.

Summing up, we did not succeed in creating an animal model of *U. urealyticum* infections, but our mice inoculation method seemed suitable for demonstrating differences in the virulence of *U. urealyticum* isolates. It is, however, questionable whether the demonstrated difference in "mouse virulence" is in correlation with the human pathogenicity. The 17 cases in which we compared the "mouse virulence" of the isolated strains with their probable aetiological role in human diseases, seem to prove a correlation.

Some factors seemed to complicate the "mouse virulence" method. Only fresh ureaplasma isolates are suitable because the subcultured strains lost their

"mouse virulence" within a short time. Another factor is the medium "mouse virulence" of calf and swine ureaplasma isolates. Only the chicken strain was avirulent. The possible human pathogenicity of the *U. urealyticum* strains of animal origin is an unsettled question.

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ADENOVIRUS-INDUCED IMMUNE RESPONSE IN OVINE FETUSES

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Ovine fetuses were examined for immune responsiveness to ovine adenovirus (strain ORT/111) at 70–80 days of gestation. Nineteen days after experimental infection, all infected fetuses, along with their eventual non-infected twins, had neutralizing serum antibodies to the virus in the titre range of 1 : 32–1 : 128. The antibodies were identified electrophoretically as subclass IgG_1 and IgG_2. No other Ig-class was involved in the immune response. Infection of the fetuses accounted for antibody production in the maternal organism, too. Immunofluorescence tests detected a periarteriolar, diffuse or focal occurrence of IgG-positive cells in the fetal spleen, and in pulmonary, renal and cotyledon tissue samples. IgM-positive cells were found exclusively in the spleen, always in a low number and in diffuse distribution. No Ig-carrier cells were detected in the liver, thymus and lymph nodes. The blood contained both IgG and IgM-positive cells at a proportion of 6–7 and 1–2%, respectively. Spleen cell cultures stimulated *in vitro* with ovine adenovirus antigen showed an average ^{3}HTdR incorporation rate of 22%.

During recent years much important new informations have been accumulated on the conditions of fetal immune response. According to present knowledge, the appearance of immunological competence is a deterministic event of the fetal maturation process, and as such it is a characteristic feature of the ontogenetic development. Specific immune responsiveness of the fetus has been observed in several species [1–6] including sheep [7–11] not only in the humoral [12] but also in the cell-mediated [9, 13] and local form [7]. SILVERSTEIN *et al.* [10] even found differences between the inducer potency of certain antigens.

In our own experiments we pursued the development of immune response in ovine fetuses artificially infected with adenovirus. Virus neutralizing serum antibodies were examined for titre and Ig-class, the localization of antibody producing cells in lymphoid organs was established, and the response of immunocompetent cells to antigenic stimulus was followed up *in vitro*.

Materials and methods

Experimental infection of ovine fetuses. A total of 14 fetuses carried by 10 three-year-old Merino ewes, was used. At 75–80 days of gestation the ewes were laparotomized and the fetuses were infected *in utero* with a 10^{-5} $TCID_{50}$/ml suspension of the strain ORT/111, closely related with type 2 bovine adenovirus [14]. The strain was propagated in secondary fetal ovine kidney cell cultures, supplemented with 10% fetal calf serum. Part of the fetuses was infected intra-

muscularly, and part orally, through the amniotic fluid. In the case of twin pregnancy only one fetus was infected. Control fetuses were treated with virus-free tissue culture medium (Table I).

The ewes were killed by bleeding 19 days after infection of the fetuses.

Heparinized and non-heparinized blood samples were taken from the umbilical vein of each fetus after killing the ewes. Blood samples were withdrawn from the ewes before experimental infection and during exsanguination.

Virus neutralization tests were performed with 100 $TCID_{50}$/0.1 ml virus material.

Analysis of serum samples by polyacrylamide gel (PAG) electrophoresis and immunoelectrophoresis was carried out as described previously [15].

Lymphocytes were collected from heparinized blood samples by centrifugation, and were suspended at 10^6/ml density in 10% fetal calf serum (FCS) containing Hanks' solution.

Spleen and thymus cells were obtained from organ specimens by homogenization in PBS in a glass homogenizer. The coarse sediment was discarded, the lymphoid cells were separated with Ficoll-Paque, and suspended in 10% FCS containing Hanks' solution, at a cell density of 1×10^6/ml.

For immunofluorescence tests, samples of spleen, liver, thymus, lymph nodes, kidney, lung and cotyledon were fixed and embedded by San Marie's method [16]. The sections were deparaffinized, washed for 10 min each in three changes of PBS, counterstained with Evans blue (1 : 125 000), coated with 1 : 3 glycerol PBS, and covered with a coverslip.

Anti-ovine IgG and anti-ovine IgM sera were prepared as described in a previous paper [15]. The IgG fraction of the antisera was isolated by preparative purification, and was labelled with FITC (Fluka AG).

Autoradiography. Spleen cell suspension at 1.5 ml aliquots was pipetted into Leighton tubes and 0.5 ml inactivated adenovirus suspension was added to each. To the control cultures

Table I

Scheme of experimental infection of the fetuses

Serial No. of pregnant ewe	Designation of fetus	Route of infection	Dose 10^5 $TCID_{50}$/ml)
1	1/a	i. m.	0.2 ml
	1/b	—	—
2	2/a	i. m.	0.2 ml
	2/b	—	—
3	3/a	p. o.	3.0 ml
4	4/a	p. o.	3.0 ml
5	5/a	i. m.	0.2 ml
6	6/a	i. m.	0.2 ml
7	7/a	p. o.	0.2 ml
8	8/a	p. o.	0.2 ml
9	9/a	i. m.*	0.2 ml
10	10/a	p. o.*	0.2 ml
	10/b	—	—

* Tissue culture medium not containing virus

0.5 ml PBS was added instead. The cultures were then incubated at 37 °C. At 57 hr incubation 10 μCi tritial-thymidine was added to each culture. Sixteen hours later the slides were fixed in methanol, washed in PBS, immersed in Ilford-Nuclear K_2 emulsion, dried, and incubated in the dark at 4 °C for six days. After incubation, the slides were developed in A49-ORWO solution, fixed, and stained with the May–Grünwald technique [17, 18].

Results

The adenovirus-induced fetal immune response was studied by different methods. Virus neutralization test was used for detection of serum antibodies, whose Ig-class was then identified by electrophoretic procedures. Ig-carrier cells were detected in the lymphoid organs with immunofluorescence test, and autoradiography was employed for follow-up of lymphoid cell response to *in vitro* antigenic stimulus.

Virus neutralization tests. Neutralizing antibodies appeared in the sera of all virus-treated fetuses, at titre levels of 1 : 32–1 : 128 and 1 : 48, after intramuscular and oral infection, respectively.

In two twin pregnancies (ewes Nos 1 and 2), neutralizing antibodies appeared in the serum of the non-infected fetal twin, in the titre range of 1 : 16–1 : 32.

Table II

Result of virus neutralization tests

Designation of fetus	Fetal serum	Maternal serum	
		Preinfection titre	Postinfection titre*
1 a	1 : 32	1 : 4	1 : 128
b	1 : 32		
2 a	1 : 128	1 : 8	1 : 128
b	1 : 16		
5 a	1 : 64	1 : 4	1 : 192
6 a	1 : 48	1 : 8	1 : 128
7 a	1 : 48	1 : 8	1 : 64
8 a	1 : 64	1 : 4	1 : 128
9 a	1 : 2	1 : 2	1 : 2
10 a	1 : 2	1 : 2	1 : 2
b	1 : 2		

a = experimentally infected fetus
b = twin, not infected
* 19 days postinfection

The neutralizing antibody titre of the maternal sera increased over the preinfection level, as shown in Table II. The control fetuses had no serum antibodies to adenoviruses. Ewes Nos 3 and 4 aborted their fetuses on the seventh day after infection of the latter through the amniotic fluid.

Electrophoretic analysis. Immunoelectrophoretic separation showed the presence of IgG in the sera of all infected fetuses. There was a double IgG precipitation arc in each case, including the twins designated with "b". No other Ig-class was detected. The sera of the control fetuses contained no immunoglobulins.

Polyacrylamide gel electrophoresis showed the presence of IgG in the sera of both infected ("a") and non-infected ("b") twins.

Immunofluorescence tests. Immunofluorescent cells were detected with anti-ovine IgG conjugate in the spleen tissue of all infected fetuses including the "b" twins. The Ig-carrier cells showed three different patterns: in some cases they occurred for the most part around vessels (Fig. 1), in others diffusely (Fig. 2), while in Cases 2a and 5a both focally and perivascularly (Fig. 3).

Fluorescent cells were also seen in the renal glomeruli, usually in low, less often in high, numbers (Fig. 4).

IgG-positive cells occurred in the lung tissue chiefly around vessels, but sporadically also in peribronchial localization. The lung tissue of one orally infected fetus (No. 7/a) showed a particularly intensive fluorescence.

The cotyledon contained Ig-carrier cells also in its fetal compartment.

No Ig-carrier cells were detected in the liver, thymus and lymph nodes.

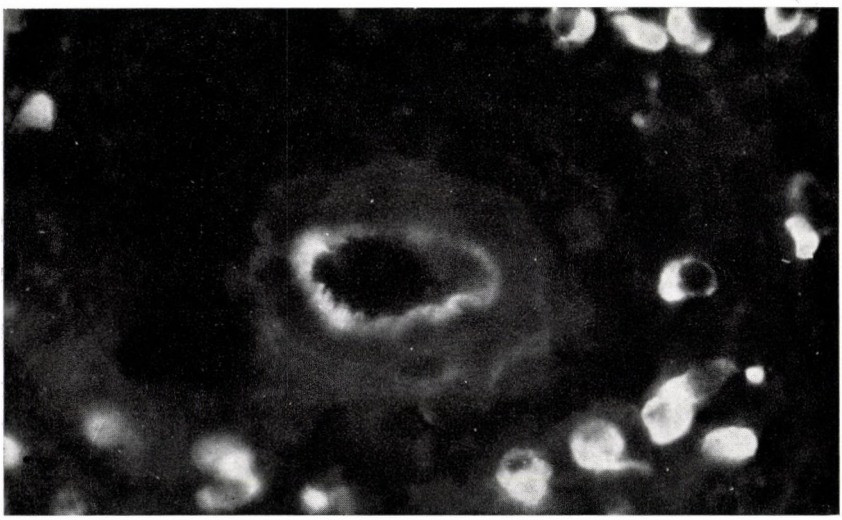

Fig. 1. IgG-carrier cells in periarterial localization (spleen)

In blood smears, 6–7% of the cells showed fluorescence; a similar percentage was found in smears from "b" twins. No fluorescent cells occurred in the tissue and blood samples of the control fetuses.

Cells responding to anti-ovine IgM conjugate occurred exclusively in the fetal spleen, always in a low number and in diffuse localization. In the blood smears, 1–2% of the cells were anti-ovine IgM-positive.

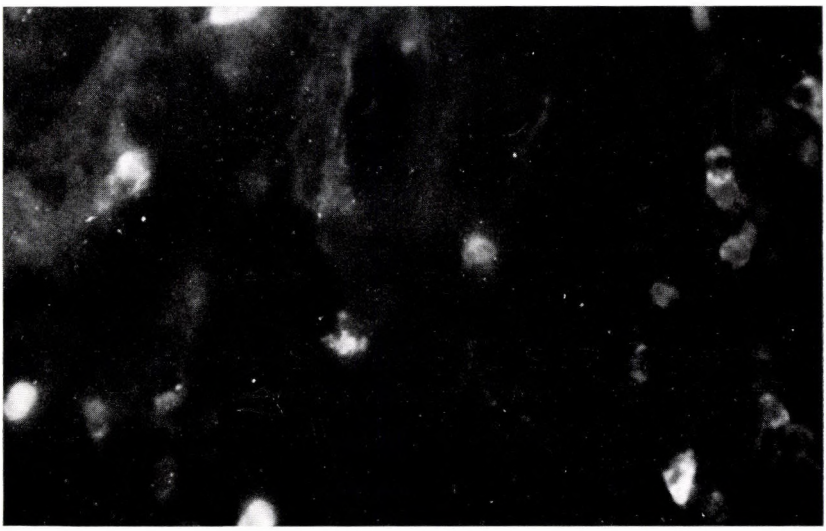

Fig. 2. Diffusely distributed IgG-carrier cells (spleen)

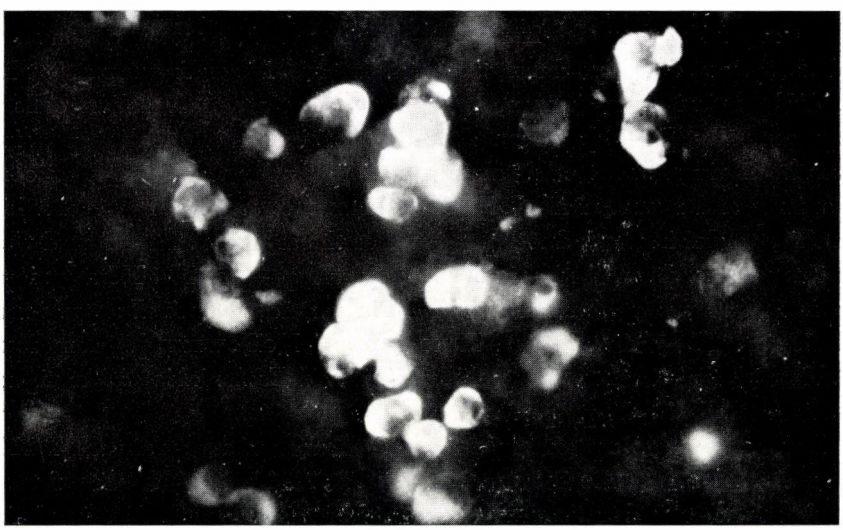

Fig. 3. Focal distribution of IgG-carrier cells (spleen)

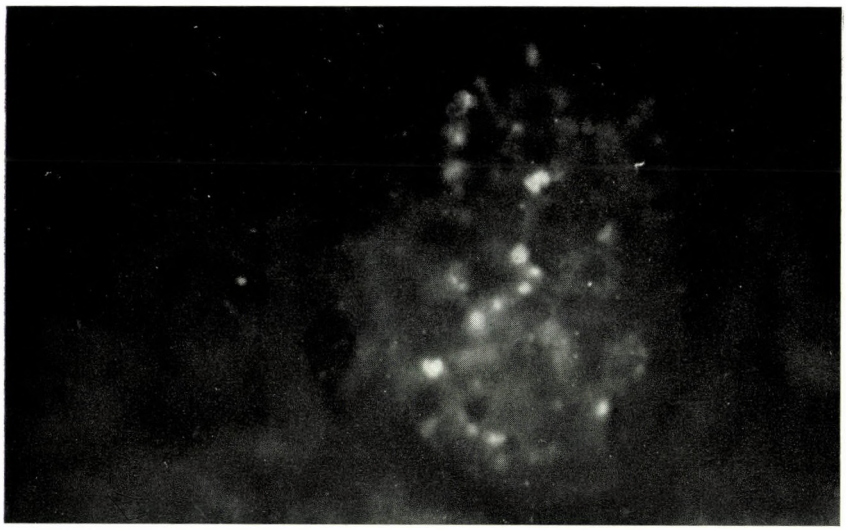

Fig. 4. IgG-carrier cells in a renal glomerulus

Autoradiography. ^{3}HTdR-incorporation of antigen-stimulated spleen cells in cultures from intramuscularly infected fetuses and "b" twins was 22% on the average. The orally infected fetus No. 7/a showed a much lower (9%) ^{3}HTdR incorporation rate.

Discussion

Investigations into the laws of immunogenesis led about two decades ago to the recognition that the immunological responsiveness of the susceptible lymphoid system develops during intrauterine life. According to present knowledge, differentiation of immunologically competent mammalian cells takes place prenatally [19–23], and the ability of immunological responsiveness evolves in a given stage of fetal maturation, under the control of complex mechanisms and interactions [24]. SILVERSTEIN *et al.* [9] observed a kind of hierarchical order in the inducer potential of the antigens. They found that the ovine fetus responds earliest—at 66–70 days of age—to the φX bacteriophage, while it is still irresponsive to diphtheria toxoid, *Salmonella typhi-murium* and BCG. In older ovine fetuses, HUSBAND and MCDOWELL [7] demonstrated a local immune response to *S. typhi-murium* and *Escherichia coli* antigens administered on the 30–17 prepartal days.

In the present experiments the ovine adenovirus strain ORT/111 was found to induce a specific immune response in 70–80-day-old ovine fetuses, to judge from the appearance of virus-neutralizing IgG-antibodies in their sera and different organs. The antibodies found represented both subclasses of IgG

(IgG_1 and IgG_2), but no other class was detected by immunoelectrophoresis. This is in accord with the observation of FAHEY and MORRIS [25] that less than 87-day-old ovine fetuses failed to produce 2-mercaptoethanol-sensitive antibodies. In the terminal stage of intrauterine life, between the 30th and 17th prenatal days, HUSBAND and MCDOWELL [7] observed the synthesis of IgA and IgM-type antibodies in the course of local immune response induced with a corpuscular *E. coli* antigen.

Infection of the fetuses with adenovirus accounted for a significant increase of the maternal neutralizing serum antibody titre over its preinfection level. It follows that either the adenovirions themselves, or the mediator substances produced by the fetal lymphoid system gained access to the maternal organism by the transplacental route. HUSBAND and MCDOWELL [7] reported that administration of a bacterial antigen to the ovine fetuses enhanced IgG production in the maternal organism. But ROSSI *et al.* [13] found no significant increase of the maternal immune response on treatment of bovine fetuses with *Brucella abortus*, *Mycobacterium bovis* and tetanus toxoid.

Further on, we investigated the occurrence of Ig-carrier cells in samples of fetal spleen, liver, thymus, lymph nodes, lung, kidney and cotyledon, using anti-IgG and anti-IgM conjugates. The Ig-positive cells of the spleen were also examined for topographic distribution. Being the largest lymphoid structure, the spleen plays a decisive role in development of the immune response [26–29]. Development of the immunologically competent splenic structures is, however, poorly understood. WEISSMAN [30] observed that the ring-like periarteriolar lymphocyte aggregations found in the fetal mouse spleen contained Ig-positive cells, but thymus-derived cells (T-lymphocytes) appeared in them only post partum. We found a dissimilar topography of Ig-carrier cells in the fetal ovine spleens: the Ig-positive cells were localized around arterioles in most cases, but less often they showed a focal — germinative centre-like — distribution. IgG-positive cells were not found in the fetal liver, lymph nodes and thymus, but they were detected in the lung, renal glomeruli and cotyledon. IgM-positive cells occurred exclusively in the spleen, always in diffuse localization. IgG and IgM-positive cells were equally present in blood smears, at proportions of 6–7 and 1–2%, respectively.

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IMMUNOLOGICAL PROPERTIES OF STAPHYLOCOCCUS AUREUS COAGULASE

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(Received January 9, 1979)

Using purified enzyme in agar gel precipitation, it was shown that staphylococci produce two types of coagulase different in biological action and immunological property. The homologous enzyme is specific for human fibrinogen in strains belonging to phage groups I, II, III and specific for bovine fibrinogen in strains of phage group IV. The heterologous enzyme acts upon bovine fibrinogen in case of strains belonging to phage groups I, II or III, and upon human fibrinogen if they belong to phage group IV. The complete enzyme produces with the serum of the homologous enzyme two precipitation bands in agar gel; one is unidentical, the other identical with the coagulase of strains belonging to any of the phage groups. In a heterologous system, the enzymes of every strain give a single band reaction. The strains isolated from bovine mastitis and typable with phage 116 of phage group II are an exception in that they give a precipitation band in both the homologous and the heterologous systems. These staphylocoagulases show in both systems antigenic identity with the homologous coagulase produced by strain of phage group IV.

Colonization of *Staphylococcus aureus* and development of the pathological process depends on the fact whether the strain is capable to ensure its own protection before being recognized by the macrophages. In *S. aureus* strains, this protection develops under the effect of coagulase production. Therefore, the activity of the enzyme and its immunological properties are of importance as to the fate of staphylococci in the host. Several authors connect the coagulase of *S. aureus* with pathogenicity [1—4], others also with virulence [5—10].

A number of workers investigated the antigenic properties of staphylococcal strains belonging to different phage types. RAMMELKAMP *et al.* [11] and DUTHIE [12] described three antigenic types among human, and one antigenic type among bovine strains. BARBER and WILDY [13] produced hyperimmune sera in the rabbit against coagulases from staphylococci of each of the three main bacteriophage groups and against the coagulase of the strain typable with the phages 3A and 42E. They found that these sera did not affect the clotting of plasma by coagulase from other strains, but considerably decreased the activity of the autogenous coagulase.

BLOBEL and BERMAN [14] in their adsorption experiments with hyperimmune sera containing anticoagulases found coagulases of different antigenicity in the strains of the four phage groups.

When titration of coagulase was done parallel in human and bovine plasma in experiments with strains of human and bovine subspecies, I found that one and the same strain produced simultaneously two types of coagulase different in action and quantity. Strains of phage groups I, II and III produce beside a human fibrinogen-specific (homologous) enzyme also a bovine fibrinogen-specific (heterologous) enzyme. A similar relationship was found in strains of phage group IV, where the enzyme clotting bovine fibrinogen is the homologous and the enzyme acting on human fibrinogen is the heterologous one.

In further studies I wanted to clarify the immunological properties of the enzyme. Since my previous investigations proved that intermediary strains lysed by phage 116 of phage group II often cause bovine mastitis in Hungary, examination of the coagulase of these strains formed part of the study.

Materials and methods

Production and titration of immunologically pure coagulase. The strains were propagated in nutrient broth of pH 7.2. The cell-free filtrate was concentrated 10-fold, then chromatographed on G-75 with 0.4F phosphate citrate buffer. The fractions were titrated in a corresponding clotting system (human or bovine plasma, 20% phosphate citrate buffer pH 7.0, 1 vol% bovine serum albumin). Enzyme purity of the coagulase-containing fractions was checked by acryl amide gel and immunoelectrophoresis. Haemolysin content of the fractions was adsorbed on sheep erythrocyte stroma. The immunologically pure enzyme was adjusted so as to coagulate equal plasma volumes.

Vaccine production. The pure enzyme of the above titre was mixed with an equal amount (200 ml) of homologous or heterologous citrated plasma. After clotting, the precipitate was centrifuged at 3000 rpm for 30 min than allowed to dry at room temperature. The rabbits were immunized with doses containing 200 mg of the vaccine suspended in 2 ml incomplete Freund adjuvant.

Serum production. The rabbits were immunized on three occasions in weakly intervals before taking the first test blood sample. Antibody titre of the serum was determined with the purified enzyme antigen.

Examination of the identity of coagulases of strains of different phage groups. This was done by agar gel precipitation in 2 systems, (a) homologous serum + complete enzyme anti-

Table I

Characteristic properties of the strains

Strain No.	Phage group	Lysing phages	Haemolysin produced	Origin
719/75	I	52 A +	$\alpha + \delta$	Human pathological
729/76	I	29 ++	$\alpha + \delta$	material
499/77	II	3A +; 71 +	$\alpha + \delta$	Human pathological
765/76	II	3C++; 55 +; 71 +	$\alpha + \delta$	material
842/75	II	116 ++	$\beta + \delta$	Bovine mastitis
774/76	II	116 ++	$\beta + \delta$	
317/76	III	42E +; 75 +; 84 +	$\alpha + \delta$	Human pathological
654/74		53 ++	$\alpha + \delta$	material
129/77	IV	102+; 117 ++; 42D +	$\alpha + \beta + \delta$	Bovine mastitis
640/75	IV	102+; 107 +; 117 ++	$\alpha + \beta + \delta$	

gen; (b) heterologous serum + complete enzyme antigen. The complete enzyme antigen of the above titre was measured into the central well and 0.02 ml volumes of the sera to be tested were measured into the wells around it.

Strains. Characteristic properties of the strains under study are shown in Table I.

Results

Comparative examinations by agar gel precipitation. In assays with homologous sera, two precipitation bands are formed if the strains belong to the same phage group. The first band is produced between the enzyme and serum of the strain belonging to the identical phage group. Exceptions are strains of phage group IV and those typable with phage 116 of group II, which react with each other, too. These sera exhibit complete identity. The other band develops between the enzymes and the sera of every strain; these reactions display complete antigenic identity (Fig. 1.). In assays with heterologous sera there is a single-band reaction showing complete antigenic identity between

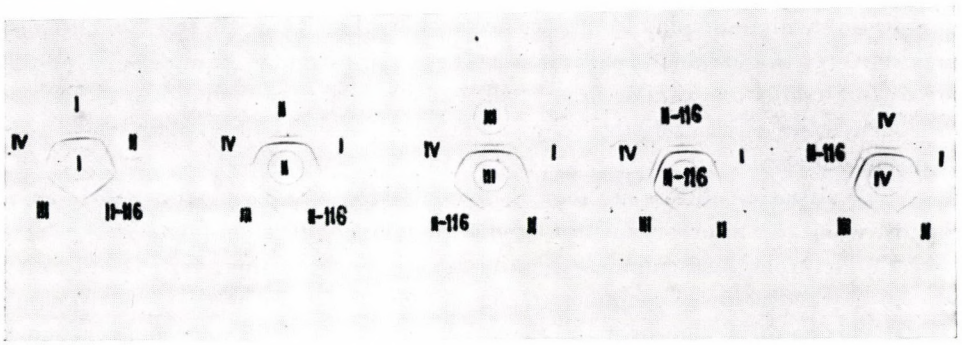

Fig. 1. Examination in agar gel of the identity of complete enzyme antigen and homologous sera

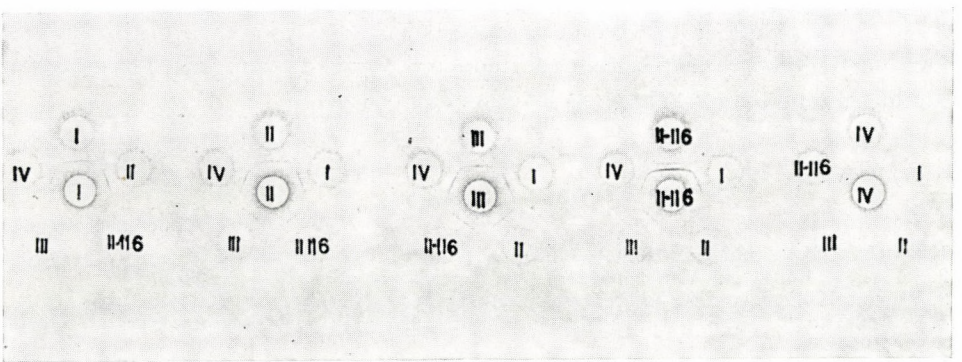

Fig. 2. Examination in agar gel of the identity of complete enzyme antigen and heterologous sera

the coagulases and the sera of the strains of every phage type, except the strain phage type 116 of phage group II, where the reaction is double-banded (Fig. 2).

Discussion

The examinations indicate that *S. aureus* strains produce two coagulases identical in molecular weight but different in biological action and immunological properties. The strains belonging to the individual phage groups utilize both coagulases to clot homologous fibrinogen, i. e. when colonizing the same organism. This is indicated by the double reaction observed in homologous systems between the antigen and the serum of the strain belonging to the same phage group. On the other hand, and in agreement with the results of BLOBEL and BERMAN [14] coagulases of the strains belonging to the individual phage groups differ in antigenic properties. The present studies suggest that the difference is valid only for the homologous coagulases of the strains. Exceptions are the homologous coagulases of the strains of phage group IV (bovine subspecies) which display complete antigenic identity with the homologous coagulases of the strains typable with phage 116 of phage group II. Although the latter strains are similar in several aspects to strains belonging to human subspecies, the characteristics of coagulase activity might explain their role in bovine mastitis.

The situation differs as concerns the clotting of heterologous fibrinogen, that is, when the microorganism is about to colonize an alien host. In this case only the heterologous coagulase participates in the reaction. This is indicated by the markedly lower coagulase titres of the strains in heterologous plasma [7, 8]. This could account for the fact that, owing to their enzyme specificity, strains of certain subspecies are less capable to colonize the heterologous organism.

From among the great number of strains tested, those typable with phage 116 of phage group II are certainly exceptions, since their entire enzyme stock participates in clotting both human and bovine fibrinogens. These strains should therefore be regarded as intermediary forms.

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STAPHYLOCOCCUS AUREUS HAEMOLYSINS: THEIR USE IN STRAIN TYPING

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(Received January 9, 1979)

By using a β-lysin-producing test strain and anti-$\beta + \delta$ lysins, the lysins of *Staphylococcus aureus* strains can easily be determined. The method was used for examination of the haemolysin production of 300 *S. aureus* strains isolated from human post mortem material and bovine mastitis. The results were cross-checked with those of three other typing tests, viz. phage typing, crystal-violet activity and lipase production. Strains isolated from human material produced most frequently $\alpha + \delta$, and less frequently δ or α lysins. The isolates belonged to phage groups I, II or III, were crystal violet negative and produced lipase. The bovine strains produced mostly $\beta + \delta$ or $\alpha + \beta + \delta$ lysins, less frequently δ or β lysins. The isolates belonged to phage group IV or were typable with phage 116 of phage group II. The majority of the strains was crystal-violet negative, lipase production was not characteristic.

Staphylococcus aureus strains produce three different haemolysins: α, β, δ. Originally, staphylococci formed all the three haemolysins (thereafter lysin). In the course of mutations, however, several combinations emerged through the loss of the ability to excrete one or two toxins [1, 2]. This fact can be made use of in the typing of strains. According to MEYER [3], determination of lysins is a valuable tool in the typing of *S. aureus* strains. This author used a $\beta + \delta$-lysin-producing test strain for the demonstration of lysins and deduced the lysin-producing capacity of the strains from the interaction of the lysin of the test strain with those of the strains under study. Lysin production is examined on blood agar plates after incubation at 37 °C for 24 hr. The underlying principle is that β lysin inhibits α lysin, whereas it potentiates the effect of δ lysin. In the course of studying the lysin-producing capacity of a number of strains, we found that the simultaneously formed lysins affect each other and also the lysin of the test strain, therefore the method involves sources of error. On the other hand, methods not using a test strain are uncertain [4] or time consuming.

In an attempt to eliminate the sources of error, we elaborated a method by which the lysin production of the strains can rapidly be determined. A β-lysin-producing test strain and anti-$\beta + \delta$ lysin were used in the procedure.

Materials and methods

The lysins are demonstrated in blood agar medium which contains 20% defibrinated sheep blood. The freshly taken blood is centrifuged at 3000 rpm for 20 min, the serum is removed, and the red cells are resuspended to the original volume with physiological saline. One

plate allows to determine 8–10 strains. First the test strain is inoculated in the total length of the diameter using the longer edge of a flame sterilized slide. Perpendicular to this, the strains under study are inoculated with the short edge of a flame sterilized slide, in a manner that the lines of inoculation should be at 3–4 mm distance from the test strain and at 16–18 mm from each other. Then round wells 6 mm in diameter are prepared at equal distances between the strains and 0.05 ml of the anti-$\beta + \delta$ lysin serum is measured into the wells. After incubation at 37 °C for 24 hr the preparations are kept at 4 °C for 48 hr. The result is read on or after the fourth day.

Production of antilysin. A strain producing $\beta + \delta$ lysins is used. In the presence of a β-lysin-producing test strain, the strains will be characterized by a double haemolysis zone in the blood agar plates. Under the effect of the β lysin, the outer zone merges with the zone of the test strain showing α-type haemolysis. Due to the potentiation of the β lysin effect, a strong haemolytic activity of the β-type is visible in the inner δ zone. The haemolysis spreads also into the discoloured zone of the test strain.

The strain under study is inoculated into 50 ml broth, pH 7.2. The culture is rotated in a shaker at low frequency (1 20 cycles/min) in a 37 °C water bath for 24 hr, then filtered through a bacterium-retaining filter. A double amount of 5%-citrate-containing bovine plasma is given to the filtrate and the preparation is kept at 37 °C for 6 hr. The precipitate is centrifuged at 4000 rpm for 1 hr. The supernatant is concentrated to one-fourth in a dialysis bag. To this an equal amount of incomplete Freund adjuvant is added and a 2 ml dose of the mixture is used for the immunization of rabbits. The injection is given to the back of the thigh 2 cm above the heel tubercle. The procedure is performed three-times at 10-day intervals. The antilysin titre of the test blood serum obtained by heart pucture is evaluated on the basis of the inhibitory effect on the haemolysis zones of the original strain produced in the plate culture. The serum can be used if it is bivalent, i. e. it inhibits both zones to an equal extent.

Results

Corresponding to the three lysins (α, β, δ), the following seven combinations are possible in the lysin production of the strains: α, β, δ, $\alpha+\beta$, $\alpha+\beta+\delta$, $\beta+\delta$. In determination of the strain, the effect of the lysins as well as the lysin–antilysin interaction have to be taken into account.

1. If the strain produces only α lysin, the haemolysis is strong, of the β-type, the zone is broad, the edges are blurred. A wedge-shaped inhibition

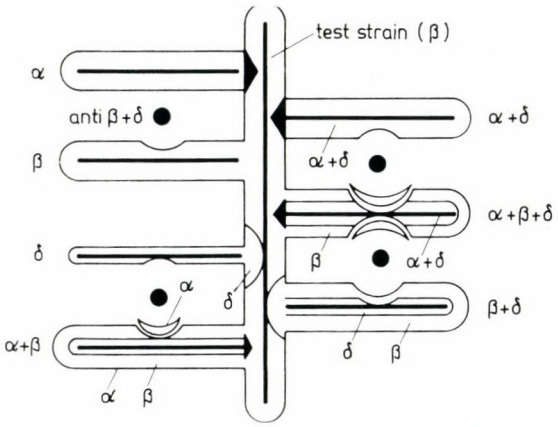

Fig. 1. Scheme for the determination of haemolysins

pattern is visible in the discoloured zone of the test strain. There is no antilysin effect (Fig. 1 and Fig. 2).

2. With β-lysin-producing strains, the haemolysis zone of the strain merges with that of the test strain. The discoloured zone is homologous, haemolysis is weak (no total clearing), the zone is sharply demarcated from its environment. On the effect of β-antilysin, inhibition can be seen in the haemolysis zone (Fig. 1 and Figs 2–3).

3. The haemolysis zone of δ lysin is usually narrow, haemolysis is approximately of β degree. There is a semicircular extensive β haemolysis in the zone of the test strain. The δ-antilysin produces inhibition (Fig. 1 and Figs 2–6).

4. In case of joint $\alpha + \beta$ lysin excretion, the haemolysis zone is doubled. Beside a circular zone, sharply demarcated from its environment and showing a weaker haemolysis (β lysin), an inner fully cleared zone is visible (α lysin).

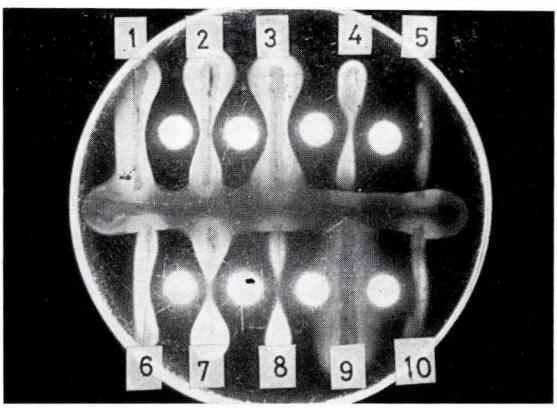

Fig. 2. α (9), δ (4, 5, 6, 8, 10) and $\beta + \delta$ (1, 2, 3, 7) lysin-producing *S. aureus* strains

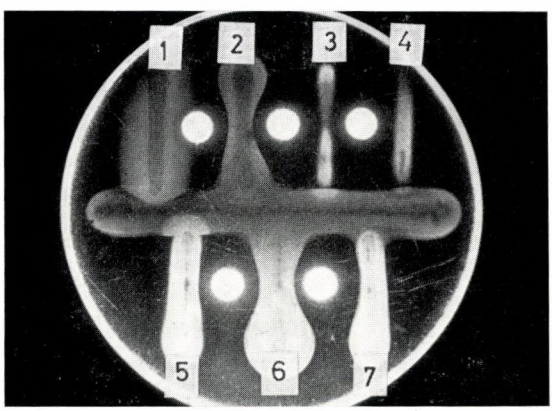

Fig. 3. β (2), δ (3, 4, 5) $\alpha + \delta$ (1) and $\beta + \delta$ (7) lysin-producing *S. aureus* strains

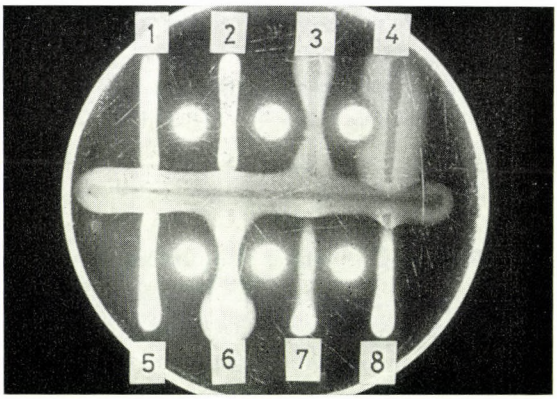

Fig. 4. β (3), δ (1, 2, 5, 7, 8), $\alpha + \delta$ (4) and $\beta + \delta$ (6) lysin-producing *S. aureus* strains

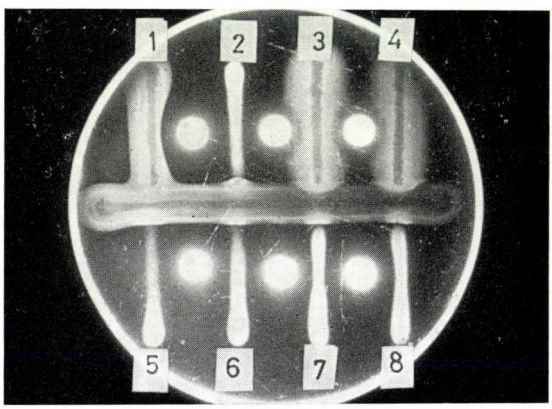

Fig. 5. δ (2, 5, 6, 7, 8), $\alpha + \delta$ (3, 4) and $\beta + \delta$ (1) lysin-producing *S. aureus* strains

The latter shows some degree of inhibition in the zone of the test strain. The antilysin effect is characteristic. The anti-β lysin of the serum inhibits the β lysin. The inhibitory action of β lysin on α lysin ceases in the zone, therefore a semilunar β-type haemolysis pattern develops under the effect of the liberated α lysin (Fig. 1).

5. If the strain produces $\alpha+\beta$ lysins, haemolysis is pronounced (β-type), inhibition of the zone of the test strain exhibits a pattern as if the strain formed only α lysin. The presence of δ lysin can be deduced from the inhibition of the antilysin effect (Fig. 1 and Figs 2–6).

6. If all the three lysins are formed, the emerging pattern simulates $\alpha+\beta$ lysin production. At the same time, the inhibitory effect of the antilysin involves also the inner ($\alpha+\delta$) lytic zone, therefore the semilunar discolouration developing on α lysin action spreads to the inner zone (Fig. 1 and Fig. 6).

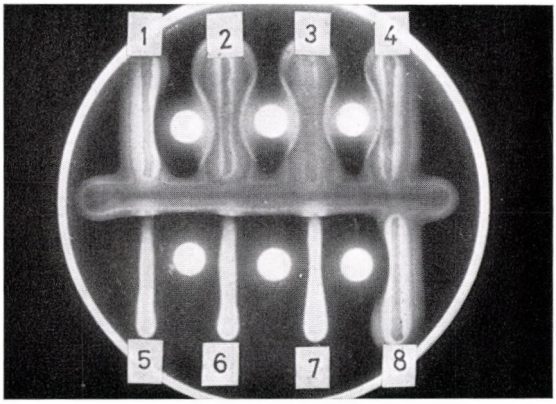

Fig. 6. δ (5, 6, 7), $\beta + \delta$ (1, 8) and $\alpha + \beta + \delta$ (2, 3, 4) lysin-producing *S. aureus* strains

7. In case of $\beta+\delta$ lysin production, the haemolysis zone is doubled. A complete (β-type) haemolysis is visible in the inner zone extending with a large halo to the haemolysis zone of the test strain. The antilysin inhibits both zones (Fig. 1 and Figs 2–6).

The method was used for the determination of haemolysin production of 300 *S. aureus* strains each isolated from human pathological materials and bovine mastitis. The results were evaluated together with those of phage typing, crystal-violet activity and lipase production [3, 5, 6] as shown in Tables I, II, III and IV.

Table I

Haemolysin production, crystal-violet activity and lipase production of 300 S. aureus strains isolated from human pathological material

Haemolysin produced	Strains		Crystal violet test		Lipase production	
	No.	%	+	−	+	−
α	24	8.0	—	24	24	—
β	3	1.0	2	1	3	—
δ	81	27.0	9	72	78	3
$\alpha + \beta$	—	—	—	—	—	—
$\alpha + \delta$	178	50.9	33	145	178	—
$\alpha + \beta + \delta$	3	1.0	—	3	3	—
$\beta + \delta$	11	3.6	2	9	10	1
Total	300	100.0	46	254	296	4

Table II

Phage pattern and haemolysin production of 300 S. aureus strains isolated from human pathological material

Phage groups (lysing phages)	Strains		Haemolysins						
	No.	%	α	β	δ	$\alpha+\beta$	$\alpha+\delta$	$\alpha+\beta+\delta$	$\beta+\delta$
I 29, 52, 52A, 80, 81, 95	89	29.7	—	—	6	—	83	—	—
II 3A, 3C, 55, 71, 116	51	17.0	16	—	21	—	10	3	1
III 42E, 47, 53, 77, 83A, 84, 85	116	38.7	8	—	23	—	85	—	—
M 3A, 3C, 29, 42E, 52A, 53, 83A, 94, 96, 117, 118	32	10.6	—	1	31	—	—	—	—
NT	12	4.0	—	2	—	—	—	—	10
Total	300	100.0	24	3	81	—	178	3	11

M = mixed
NT = non-typable

Table III

Haemolysin production, crystal-violet activity and lipase production of 300 S. aureus strains isolated from bovine mastitis

Haemolysin produced	Strains		Crystal violet test		Lipase production	
	No.	%	+	−	+	−
α	16	5.3	8	8	11	5
β	19	6.3	19	—	9	10
δ	58	19.3	50	8	31	27
$\alpha+\beta$	7	2.3	7	—	7	—
$\alpha+\delta$	23	7.7	16	7	18	5
$\alpha+\beta+\delta$	87	29.0	75	12	18	69
$\beta+\delta$	90	30.0	85	5	45	45
Total	300	100.0	260	40	139	161

Table IV

Phage pattern and haemolysin production of 300 S. aureus strains isolated from bovine mastitis

Phage groups (lysing phages	Strains		Haemolysins						
	No.	%	α	β	δ	$\alpha+\beta$	$\alpha+\delta$	$\alpha+\beta+\delta$	$\beta+\delta$
II (3A, 3C)	9	3.0	—	—	—	2	5	2	—
II (116)	78	26.0	5	2	32	—	9	14	18
III (42E, 77, 83A)	9	3.0	5	1	2	—	—	—	1
IV (42D, 102, 117)	168	56.1	—	16	23	2	—	63	64
M (42D, 42E, 77, 102, 117)	7	2.3	—	—	—	2	1	3	1
NT	29	9.6	6	—	1	1	8	7	6
Total	300	100.0	16	19	58	7	23	87	90

M = mixed
NT = non-typable

Discussion

In agreement with the data in the literature [3, 4], the results indicate that the method is well suited for the typing of *S. aureus* strains. Isolates from human pathological materials usually produce $\alpha+\beta$ lysin, less frequently δ or α lysin. In decreasing order (38, 29 and 17%), the strains belong to phage groups III, I and II, and are lysed by mixed phages in 10%. No characteristic correlation exists between haemolysin formation and phage group. The majority of the typable human strains are crystal-violet negative, but produce lipase almost without exception. The strains responsible for bovine mastitis are mostly $\beta+\delta$ or $\alpha+\beta+\delta$ producers, less frequently δ or β lysin producers. The participation of other lysin-producing strains is below 10%. The relationship between lysin production and phage group is also characteristic. Strains typable with phage 116 of phage group II form δ or $\beta+\delta$ lysins, strains of phage group IV excrete β and $\alpha+\beta+\delta$ lysins beside the toxins. The majority of typable strains are crystal violet positive. In contrast to MEYER's finding, their lipase production does not reveal any characteristic conclusion in typing.

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THE PROCOAGULANT ACTIVITY OF LEUKOCYTES PRETREATED WITH RADIODETOXIFIED ENDOTOXIN

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Rabbits were treated with *Escherichia coli* O89 endotoxin detoxified by ionizing irradiation (^{60}Co-gamma). The leukocytes (PMNs in 90%) obtained from rabbits that had been treated with the mother endotoxin elicited a well defined, those obtained from rabbits pretreated with detoxified endotoxin elicited a less pronounced, procoagulant activity. It is suggested that the procoagulant effect may play a part in the mechanism of the local Shwartzman phenomenon.

A few years ago we succeeded in preparing an endotoxin with reduced toxicity and unchanged favourable properties [1]. The detoxified endotoxin induced endotoxin tolerance, increased natural resistance, and proved to be protective against shock and radiation damage, similarly as the untreated endotoxin. It also retained its immunoadjuvant activity.

The preparation was less active than the mother endotoxin in causing leukopenia and thrombocytopenia, and in reducing the fibrinogen level. The complement level was practically unchanged while the local Shwartzman phenomenon was substantially reduced in the rabbits pretreated with the detoxified endotoxin [2–6].

Recently, it has been reported [7, 8] that a substance with well-defined procoagulant activity is released from PMN leukocytes of rabbits pretreated with endotoxin. Furthermore, a procoagulant effect was generated by human leukocytes incubated with endotoxin *in vitro* [9].

The present paper describes the effect of radiodetoxified endotoxin on the procoagulant-generating activity of endotoxin-induced leukocytes.

Materials and methods

Endotoxin and its detoxification. The endotoxin used in the present work had been extracted by phenol–water from *Escherichia coli* O89 [10] and repeatedly centrifuged at 100 000 *g* in a Beckman L2 65B ultracentrifuge. The endotoxin to be detoxified was suspended in distilled water (10 mg/ml) and irradiated by the method developed by Previte *et al.* [11] and modified by us. Endotoxin samples were irradiated with various doses, viz. 5, 10, 15 and 20 Mrads. (^{60}Co-gamma, NORATOM 3500).

Leukocytes. Leukocytes were produced in male rabbits of 2500 to 3000 g body weight. Each rabbit was given intraperitoneally 40 ml of a 7% sodium caseinate solution of pH 7.2.

After an interval of 18 hr, the abdominal cavity was washed with 80 ml of a solution containing one part 3.8% sodium citrate and 4 parts physiological saline, using plastic syringes and needles. The exudate was filtered, and centrifuged at 250 *g* for 10 min; the cells were suspended and washed twice in citrate solution of the above composition. Ninety per cent of the cells thus obtained consisted of PMN leukocytes.

Pretreatment of rabbits with endotoxin or radiodetoxified endotoxin. Rabbits were divided into 11 groups each consisting of 6 animals. The rabbits in group C (control) were given no endotoxin, those in group E_I and E_{II} received 1 LD_{30} of untreated endotoxin together with the caseinate. E_{II} rabbits were given, in addition to the intraperitoneal dose, the same dose of endotoxin intravenously, 16 hr later.

Groups R_I^5, R_I^{10}, R_I^{15} and R_I^{20} were given one 0.5 mg/kg dose of radiodetoxified endotoxin from the samples irradiated with 5, 10, 15 and 20 Mrads, respectively. Groups R_{II}^5, R_{II}^{10}, R_{II}^{15} and R_{II}^{20} were treated identically and then reinjected intravenously with 0.5 mg/kg of the respective radiodetoxified endotoxin 16 hr after the intraperitoneal injection.

The exudate was withdrawn 18 hr after the caseinate injection.

Measuring of the procoagulant activity of leukocytes. The coagulating activity of leukocyte samples was tested in normal rabbit plasma. Leukocytes were incubated in saline at 37 °C for 30 min with cautious shaking at 5-min intervals. The coagulation times were related to the recalcification time of the normal plasma, the latter being regarded as 100%. The coagulation time was determined in a Hyland–Clotek coagulometer. The procoagulant activity of cell suspensions was measured in the following system: 0.1 ml normal citrate rabbit plasma, 0.1 ml cell suspension (cell counts 2.5×10^5, 5×10^5 and 1×10^6) and 0.1 ml 0.05 M $CaCl_2 \cdot 2H_2O$.

Results

The procoagulant activity of the leukocytes is shown in Figs 1 and 2.

The procoagulant activity of control (C) leukocytes, though consistently prevalent, never exceeded 10%, even if the cell count was as high as 10^6/ml;

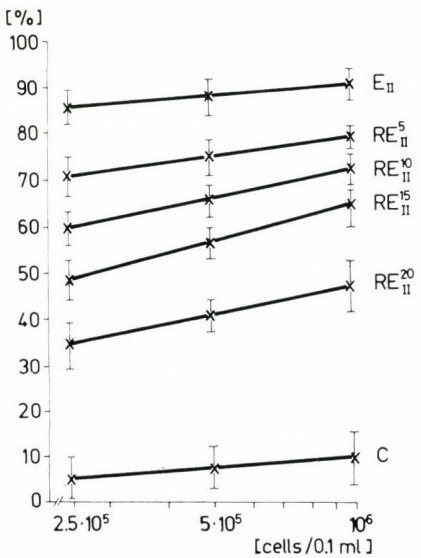

Fig. 1. Procoagulant activity of leukocytes in percentage of recalcification time. Rabbits were treated once with untreated endotoxin (E_I) and with endotoxin irradiated with various doses (RE_I)

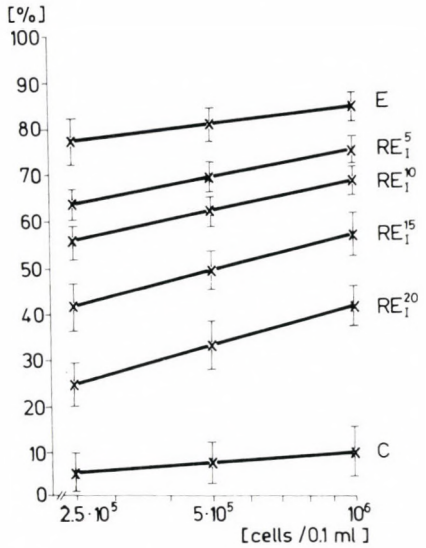

Fig. 2. Procoagulant activity of leukocytes in percentage of recalcification time. Rabbits were treated twice with untreated endotoxin (E_{II}) and with endotoxin irradiated with various doses (RE_{II})

that of the E_I leukocytes was 70–85%, i. e. the recalcification time was shortened by 70–85%. The recalcification time ranged between 100 min and 220 min.

The larger the dose of irradiation, the weaker the effect of the endotoxin on the procoagulant activity of leukocytes. The procoagulant activity inducing effect of the RE_I^{20} preparation was 1/4 to 1/3 of that of the mother endotoxin. The effect of the endotoxin on the procoagulant activity of the leukocytes was only slightly increased by the second (intravenous) dose of the endotoxin or radiodetoxified endotoxin.

Discussion

The mechanism of the procoagulant effect of leukocytes resembles the effect of the coagulating factor, in that it activates factor X in the presence of factor VII and Ca^{++} [8]. In the presence of 2.5 mM Ca^{++}, the optimum release of the procoagulant factor was observed at 37 °C between pH 7 and pH 8; the process is intensely inhibited by vinblastin and cytochalasin B [12]. Also peritoneal macrophages incubated with endotoxin elicit a pronounced procoagulant activity. The *in vitro* procoagulant activity of leukocytes from patients with chronic myelogenous leukaemia is weaker than that of normal leukocytes; leukocytes from patients with chronic lymphoid leukaemia exert practically no procoagulant activity [9].

The present results have confirmed that leukocytes (90% PMN) obtained from endotoxin-treated rabbits possess a well-defined procoagulant activity. The aim of our experiments was to show how the procoagulant activity-inducing effect of the endotoxin is changed by radiodetoxification. After an irradiation with a large dose (20 Mrads), the activity was substantially reduced. It has also been shown that a second (intravenous) injection of the endotoxin or of radiodetoxified endotoxin increases only slightly the procoagulant activity of the rabbit leukocytes.

We have reported recently [6] the Shwartzman phenomenon prepared and induced with radiodetoxified endotoxin is less intensive than the reaction induced with the mother endotoxin. In earlier experiments [13] we found that PMN leukocytes had a central role in the Shwartzman phenomenon. We may therefore conclude that among other effects [14], the procoagulant-generating effect of PMN leukocytes may play an important role in the local Shwartzman phenomenon.

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In memoriam Gyula Takátsy

The death of Dr. Gyula Takátsy on March 12, 1980 brings a sense of profound respect for his memory to all who know that he was a man of genius inventing the microtitration technique now widely used all over the world in microbiological, immunological and clinical laboratories.

He was born in Magyaratád, Hungary on October 22, 1914. He entered the University Medical School, Pécs in 1932 and graduated there in 1938. After his graduation he began to work at the Institute of Public Health, University Medical School, Pécs and from 1942 to the date of his death he was a staff member of National Institute of Hygiene, Budapest. Between 1942 and 1948 he contributed in *Rickettsia prowazeki* and other vaccine productions and in 1948 he began his career at the Department of Virology. Understandingly the laboratory facilities were very poor at that time — shortly after the World War II. There was a shortage even in basic laboratory tools like pipettes and test tubes. Just this disappointing poverty gave him an incentive to endeavour creation of "simple" tools which could replace the classical laboratory equipments. With his genuin skill the problem was solved within a very short time. Already in 1950 he published an article in Hungarian with the title "A new method for rapid and accurate performance of serial dilutions" in Kísérletes Orvostudo-

mány, describing the spiral loop instead of pipette and glass-plates with wells instead of tubes. This method, further developed by applying spiral loops with micro-volumes, plastic plates with matching well-volumes and droppers, was published in 1952 in Hungarian in the above-mentioned periodical and in 1955 in English in this journal with the title "The use of spiral loops in serological and virological micro-methods" (*3*, 191). This brilliant method did not attract considerable interest until 1962 when American authors published their results on its application. It was Dr. Takátsy's moral greatness being glad to learn that his technique has got a world-wide acceptance without respect that there has been a tendency to neglect his priority, and that he hardly had any share in profits.

Besides the micro-technique his studies on influenza virus brought him an international reputation. He made significant contributions in his 50 publications connected with variability of antigenic structure and biological properties of influenza viruses, pathological and epidemiological importance of different influenza virus variants, ecology of influenza virus, as well as influenza virus vaccine production and control.

He became the Chief of Influenza Unit at the Department of Virology in 1957 which functioned as the National Influenza Centre. Besides carrying out diagnostic work, this small Unit under his leadership produced yearly 0.5 million doses of killed influenza vaccine from 1960 on.

He was a member of the Board of the Hungarian Society of Microbiology, which Society awarded to him the "Manninger Medal" in 1979. He was honoured with high decorations several times.

He will be remembered as a scientist who greatly contributed to the advancement of research and practical work in microbiology and in related fields.

I. Dömök

EIGHTH CONGRESS OF THE HUNGARIAN SOCIETY OF MICROBIOLOGY

BUDAPEST, AUGUST 27–29, 1979

ABSTRACTS OF PAPERS

Acta microbiol. Acad. Sci. hung. 27, 199—258, 1980

Bacteriology

THE INFLUENCE OF METHICILLIN ON THE FATTY ACID COMPOSITION OF PHOSPHOLIPIDS IN STAPHYLOCOCCUS AUREUS SENSITIVE TO METHICILLIN

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The influence of methicillin (MET) at 1 μg/ml (= $^1/_2\times$MIC) and 2 μg/ml (= 1×MIC) on the fatty acid composition of phospholipids in exponentially growing *Staphylococcus aureus* 5814S was examined during a period of 2 hr treatment. In comparison with the 2 hr control, in phosphatidic acid the proportion of C12 : 3, nC12 : 0, aiC15 : 0, aiC17 : 0, aiC19 : 0, and nC19 : 0 was increased while that of nC14 : 0, nC18 : 0, and nC20 : 0 was decreased by $^1/_2\times$MIC of MET. Treatment with 1×MIC of MET resulted in an enhancement of nC16 : 0, aiC17 : 0, C18 : 1, aiC19 : 0, and nC20 : 0, whereas in a fall of C12 : 3, nC14 : 0, aiC15 : 0, and nC20 : 0. In phosphatidylglycerol the proportion of C12 : 3 and aiC15 : 0 decreased but that of aiC17 : 0 and nC20 : 0 increased under the influence of $^1/_2\times$MIC of MET. There was a striking rise in fatty acids longer than C15 while aiC15 : 0 fell on the effect of 1×MIC of MET. In cardiolipin $^1/_2\times$MIC of MET caused an enhancement in C16 : 1 and nC20 : 0 and a decrease in nC14 :0, aiC15 : 0, and nC16 : 0. Treatment with 1×MIC of MET resulted in a fall of aiC15 : 0 and nC20 : 0 and an increase in nC13 : 0, nC14 : 0, nC16 : 0 and i-C24 : 0. In lysylphosphatidylglycerol the proportion of aiC15 : 0, nC18 : 0 and nC20 : 0 was increased while that of nC16 : 0 and aiC17 : 0 was lowered by $^1/_2\times$MIC of MET. Exposure to 1×MIC of MET brought about a rise of aiC15 : 0, aiC19 : 0, nC19 : 0, and nC20 : 0 and a fall in nC16 : 0 and aiC17 : 0 acids. These results indicate that phospholipid synthesis may be one of the sites of action of MET.

MOUSE VIRULENCE OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS

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The mouse virulence of a methicillin sensitive (MS) substrain and a methicillin resistant (MR) mutant both derived from a natural isolate of *Staphylococcus aureus* 5814 of mixed population were compared. Stationary phase cultures were centrifuged and washed twice with physiological saline solution. Then the bacteria were resuspended in Fluidex-40 solution and injected intraperitoneally into white male mice of BALB/c weighing 18—22 g. After ten days, the mice were sacrificed and the kidneys and the spleen were examined macroscopically. Nine mice out of ten, injected with 2.1×10^{10} cells of MS *S. aureus* 5814S, died within one day. One mouse out of ten injected with 5×10^9 and 1×10^9 cocci died. In the survivors the spleen was

enlarged and unilateral kidney abscesses occurred. After the injection of 5×10^8–10^7 bacteria all mice survived and there was no alteration at autopsy. Nine mice out of ten injected with 2×10^{10} cells of MR *S. aureus* 5814R died within one day. After injecting 4×10^9 cocci only one mouse died. A dose of 5×10^9 cocci killed two mice, and the eight survivors showed an enlarged spleen and kidney abscesses. Whereas 5×10^8 cocci killed one mouse and there was no macroscopic change in the rest, 1×10^8–1×10^7 cocci caused no alteration. Mouse virulence of the MR and MS cocci, as regards the two strains examined, seems to be very similar. In contrast with some literary data the MR cocci proved to be pathogenic for mice.

EPIDERMOLYTIC TOXIN PRODUCTION BY STAPHYLOCOCCUS AUREUS

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Epidermolytic toxin or exfoliatin is a staphylococcal exotoxin described recently as the cause of staphylogenous toxic epidermolytic necrolysis or scalded skin syndrome in small children. Infection with toxin producing staphylococci may result in grave generalized exfoliative disease, scarlatiniform rash or bullous impetigo. Exfoliatin production of *Staphylococcus aureus* strains isolated from clinical specimens was studied by agar-gel precipitation. In generalized exfoliative disease all staphylococci isolated from nose, throat or skin lesion belonged to phage group II and produced exfoliatin. In other types of skin lesions about 50% of phage group II strains, while only about 20% of other phage group strains were exfoliatin positive. About one fifth of phage group II but only a few of other phage group staphylococci deriving from other clinical specimens, produced toxin. While exfoliatin was always detectable in the culture filtrate of strains isolated from toxic epidermolytic necrolysis, toxin production was also relatively frequent by strains deriving from miscellanous dermatological material. Our results, in accordance with literary data show that there is a close relationship between sensitivity to phage group II phages, epidermolytic toxin production and the dermal origin of the organism.

CHARACTERIZATION OF STAPHYLOCOCCUS AUREUS STRAINS ISOLATED FROM THE BOVINE MAMMARY GLAND

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Bovine mammal staphylococci were tested for cultural properties, phage pattern, enterotoxigenicity, Mayer's crystal violet test, pigment production, lecithinase activity, coagulase test with bovine and rabbit serum, thermostable deoxyribonuclease test and antibiogram. Out of the isolates 82% showed intermediate properties between the bovine and human type. Six of the intermediate and 5 of the bovine type strains produced enterotoxin (total, 11%). In the absence of clinical mastitis, only non-enterotoxigenic staphylococci were isolated.

AGGLUTINABILITY, IMMUNOGENICITY AND AGGLUTININ BINDING CAPACITY OF ACINETOBACTER STRAINS

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Living cultures of 110 *Acinetobacter* strains of different serotypes showed specific agglutination, 6 were autoagglutinable in homologous immune sera prepared with formalinized bacteria. Out of saline suspensions heated for 1 hr at 100 °C 3.4% did not agglutinate, nearly 37% showed K-type, 51% O-type and 12% a very finely granular (R-type) agglutination. Bacteria heated for 2.5 hr at 100 °C generally agglutinated in high titre, except a few low titre reactions and fine type of agglutinations. Almost in 50% of the cases the bacteria treated for 2.5 hr at 121 °C agglutinated in high titre. In sera prepared by bacteria heated for 1 hr at 100 °C, with few exceptions the living cultures agglutinated well, but the working dilutions of sera for slide agglutination were lower than those of the sera prepared by formalinized bacteria. Bacteria heated for 1 hr or 2.5 hr at 100 °C generally agglutinated in high titres, but with autoclaved suspensions the titres were lower. In sera prepared by immunization with bacteria heated for 2.5 hr at 100 °C, only one third of homologous living bacteria agglutinated at 1 : 5 dilution. The suspensions treated at 100 °C or at 121 °C usually reacted at low titre, but partly gave high titre or failed to react. In the sera prepared with bacteria autoclaved for 2.5 hr at 121 °C, with the exception of some low-titre reactions, neither the heated nor the living cultures reacted. The O and K agglutinin-binding capacity of *Acinetobacter* cultures was of high degree in the living as well as in the heated state (100 °C for 1 hr or 2.5 hr). The agglutinin-binding capacity of autoclaved bacteria was somewhat decreased. The heat resistance of *Acinetobacter* antigens varied greatly in respect to the three serological features.

INCIDENCE OF ACINETOBACTER IN FARM ANIMALS AND THEIR ENVIRONMENT

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A total of 42 *Acinetobacter* strains were isolated from 198 samples. *Acinetobacter* was isolated besides *Bordetella bronchiseptica* from the nose of a pig with atrophic rhinitis (4 strains). Environmental investigations in the breeding farm yielded 23 strains from 155 samples, different in antigenic structure from the pig strains. In a poultry breeder 2 *Acinetobacter* strains were isolated from 6 incubators. *Acinetobacter* was found besides *Streptococcus equi* in the snout of a horse which suffered from acute adenitis equorum (1 strain). It could be isolated from the snout, conjunctivae and drinking water even after recovery of the horse (4 strains). Eight strains were grown from the drinking water of animals in three farms and from a diluted manure in another farm (19 samples). Half of the strains differed in antigenic structure from Hungarian strains of human origin.

A NEWLY RECOGNIZED THERAPEUTIC EFFECT OF PRENYLAMINE

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Prenylamine, a drug applied in cardiology, has gained a new area of application in the treatment of lactose intolerance frequently occurring after infections. The drug is beneficial also in alimentary allergy and some dermatological diseases. It acts in two ways. (i) The drug attaching to the mucous membrane of the duodenum decreases its adsorptive capacity. (ii) Because of its antibacterial effect, the drug inhibits the lactose-decomposing activity of microorganisms in the duodenum, primarily the fermentation by *Escherichia coli* and *Streptococcus faecalis*. The drug also acts on *Staphylococcus aureus*, *Streptococcus pyogenes*, diphtheroids and *Candida albicans*. *Pseudomonas aeruginosa* and *Giardia lamblia* were resistant to prenylamine. The drug eliminated the symptoms for a considerable period, and caused no significant side-effects.

DIFFERENCE IN THE VIRULENCE OF UREAPLASMA UREALYTICUM ISOLATES

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The virulence of *Ureaplasma urealyticum* strains was tested by infecting white mice of undefined, LATI (Gödöllő, Hungary) and BALB/c breed intrarenally. Freshly isolated human strains differed in virulence. On subculturing in U-9 medium they showed a gradual loss of virulence. *Ureaplasma* strains of calf and swine origin were virulent to mice in a medium degree. Some of the inoculated mice showed signs of latent infection.

DETECTION OF UREAPLASMA FROM TURKEY SEMEN

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Forty-two turkey semen samples from two turkey flocks examined culturally contained *Ureaplasma* in 47.6%. Serum samples collected from the same flocks contained antibodies against the isolated *Ureaplasma* strains in 60–100%. By electron microscopic examination strain T-1001 showed an ultrastructure characteristic of ureaplasmas. By biochemical and serological features the strain differed from human serotypes I-VII, 3 cattle, 2 sheep and 1 swine *Ureaplasma* reference strains. At the same time it gave a significant cross-reaction with strain T-916 of chicken origin. By infection of turkeys and chickens of various ages strain T-1001 caused peritonitis and airsacculitis as well as antibody production.

ULTRASTRUCTURE OF TREPONEMA HYODYSENTERIAE

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By negative staining and embedding of colon and agar cultures, *Treponema hyodys enteriae* was similar in ultrastructure to other spirochetes. The undulate protoplasma cylinder is coated by a three-layered cytoplasmic membrane joining to 8–11 axial fibrilla starting at both ends of the organism. The cells are coated by a three-layered cover. Breaking of the axial fibrilla causes straightening of the cell. The broken fibrilla and the elongation of the coat resemble flagella although the organism is non-flagellated. In agar cultures great numbers of "large bodies" containing protoplasmic cylinders are seen. In the number of fibrilla, the characteristics of movement and staining properties *T. hyodysenteriae* resembles members of the genus *Borrelia*, whereas according to biological properties such as temperature requirement, localization in the host, course of the disease and the lack of arthropode vector in transmission, it should be classified in the genus *Treponema*.

AETIOLOGY OF PAEDIATRIC BACTERIAL VULVOVAGINITIS

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Vaginal samples from 0–18-year-old female patients with vulvovaginitis of non-mycotic and trichomonad aetiology were examined. Out of 123 samples 119 contained bacteria such as enterobacteria, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Haemophilus influenzae* or corynebacteria.

VALUE OF THE SUCKLING MOUSE TEST IN HEAT-STABLE ESCHERICHIA COLI ENTEROTOXIN DETECTION

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Instead of the expensive pig intestinal loop test which involves surgical intervention, we used the suckling mouse test described by DEAN *et al.*, and modified by JACKS and WU for detection of heat-stable *Escherichia coli* enterotoxin. A total of 65 strains was tested for enterotoxin production; of these 13 and 52, respectively, had been isolated from outbreaks of enteritis in suckling and weanling pig groups. Crude ST preparations were tested simultaneously in suckling mice and ligated pig intestinal loop, for induction of fluid accumulation. Among the isolates derived from suckling and weanling enteritis only 15.3 and 32.6%, respectively, proved to be positive in the suckling mouse test, 29.2% of all strains were positive in both mouse and pig loop test, and 70.7% only in the latter. As to the serotype distribution of the enterotoxic property, ST-producers were numerous in certain types, but few or missing in others. Although the suckling mouse test is not in itself sufficient for the identification of ST-producing enterotoxic *E. coli* strains, it is an excellent bioassay in the course of the ST purification procedure, if the strain produces suckling mouse positive crude ST.

PURIFICATION OF HEAT-STABLE ESCHERICHIA COLI ENTEROTOXIN (ST)

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Escherichia coli ST enterotoxin was isolated from the fluid phase of the soft agar culture of the enterotoxic type O138 *E. coli* strain No. 2023, known to produce exclusively ST. The fluid phase of a sterile soft agar medium was used as control. The crude ST preparations were heat-treated, passed through Amicon PM-10 and UM-2 membranes, and the UM-2 retentate was treated with methanol-chloroform. The aqueous phase was concentrated and purified further by immunoadsorption, filtration through Sephadex-G25 gel, and chromatography on anion exchange resin. The purified ST-preparations were then tested for biological activity in suckling mouse test and ligated pig intestinal loop. The purified ST preparation proved to be biologically active.

ISOLATION OF THE HEAT-STABLE ESCHERICHIA COLI ENTEROTOXIN (ST) BY IMMUNOADSORPTION TECHNIQUE

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Mono- and bivalent immune sera containing anti-ST antibodies were prepared in rabbits and pigs by employing carrier proteins and co-polymerisation with glutaraldehyde. Crude ST preparations were obtained by culturing the No. 2023, type O138 enterotoxic pig strain of *Escherichia coli* in soft agar. The serum immunogolobulin fraction was adsorbed onto Sepharose 4B column activated with cyanobromide. Tris–NaCl buffer (pH 8.0) was used for elution. Desorption was satisfactorily efficient in a buffer of low pH (3.0), but it was notably improved if the low pH (3.0) was combined with an increased ionic strength by adding 0.01 M Tris and 6 M urea to the buffer. After adjustment of the osmolarity, this fraction exerted a dual effect in suckling mouse tests, having accounted for fluid accumulation in the small intestine and simultaneously for clinical symptoms and gross lesions indicative of transmucosal penetration of the toxin. The toxic factor responsible for fluid accumulation could be separated by anion exchange chromatography (Bio Rad AGl-X4). The hapten-like ST enterotoxin stimulates specific antibody production under appropriate experimental conditions, and the crude ST toxin produced by the strain studied appeared to be heterogeneous.

PURIFICATION OF HEAT-LABILE ESCHERICHIA COLI ENTEROTOXIN (LT)

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For the purification of the protein-like enterotoxin LT, a product of enterotoxic *Escherichia coli* strains, a simple and efficient procedure has been developed. A cell lysate prepared from the type O141 enterotoxic *E. coli* strain SWP99 as proposed by Smith and Gyles was

fractionated with ammonium sulphate, chromatographed by immunoadsorbent technique, and desorbed. The eluted peak, which accounted for a positive reaction in the rabbit intestinal loop test, was then checked for purity by vertical polyacrylamide gel electroprohesis and immunodiffusion. Its estimated molecular weight proved to be about 75 000 dalton on analysis by polyacrylamide gel electrophoresis. The presumably heterologous nature of crude LT and the possible existence of a biologically inactive LT-like material are discussed.

PURIFICATION OF HEAT-LABILE ESCHERICHIA COLI ENTEROTOXIN (LT) BY AFFINITY CHROMATOGRAPHY

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Heat-labile *Escherichia coli* enterotoxin was isolated by immunoadsorbent technique, using cyanobromide-activated Sepharose 4B. The cell lysate of the type O141 reference strain SWP99 was used for the production of crude LT, and the lysate of the biologically inactive standard *E. coli* strain F11 was used as control. Rabbit or pig antisera prepared with purified LT or LT + ST antigen were adsorbed onto the activated Sepharose 4B column. The partly purified LT antigen, fractionated with ammonium sulphate, was eluted from the column in 0.2 M Tris + 0.5 M NaCl-containing buffer (pH 8.0), and was desorbed in 6 M urea-containing buffer (pH 7.4). The desorbed peak displayed high biological activity characteristic of LT, i.e. it showed a strong positive reaction in ligated rabbit intestinal loop. The LT-desorbing ability of 6 M urea had previously not been observed; its great advantage over other desorption procedures is its non-interference with the biological activity of LT.

ELISA FOR MEASURING VIBRIO CHOLERAE AND ESCHERICHIA COLI HEAT-LABILE TOXINS AND TOXIN-SPECIFIC ANTIBODIES

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In Dynatech Microelisa plates sensitized with purified anti-cholera toxin and with anti-*Escherichia coli* enterotoxin, cholea toxin (CT) and *E. coli* enterotoxin (LT) could be detected with a sensitivity comparable to that of CHO cell culture. In plates sensitized with toxins the titre of antitoxins could also be determined. Concentrations of toxins or antitoxins were proportionally related to the colour developed by the reaction between conjugate and substrate. Absorbance could accurately be registered by a photometer. Neutralisation test on CHO cells revealed a close antigenic relationship between CT and LT. When the plates were sensitized with toxins, ELISA confirmed this relationship, though the heterologous titres were somewhat lower than the homologous ones. When the plates were sensitized with anti-LT-IgG, both LT and C could be detected with high sensitivity. Sensitization with anti-CT-IgG resulted in a very weak reaction with LT. ELISA seems to be a sensitive, economic and simple technique for detecting toxins and measuring the potency of antitoxins.

ENTEROTOXIN PRODUCTION OF YERSINIA ENTEROCOLITICA ISOLATED IN HUNGARY

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Eighteen strains of freshly isolated *Yersinia enterocolitica* (serogroup O3) were tested. When cultured at 25 °C, they produced ST type enterotoxin detectable in suckling mouse. Strains kept in the laboratory for a longer period failed to produce toxin. Filtrates and sonicated samples of the strains exerted no LT activity in rabbit skin (PF test). The ST nature of the enterotoxin was identified by ligated rabbit loop test and by the failure of toxin production at 37 °C. Toxin production was influenced by the media used. For example *Y. enterocolitica* produced toxin in Casamino but not in Sakazaki medium. Invasiveness of the bacteria was assayed in Serény's test: all were negative, only three strains caused mild conjunctivitis. In accordance with literary data, none of the strains utilised rhamnose.

TOXINS OF VIBRIO ENTERITIDIS (NAG VIBRIOS)

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Vibrio enteritidis strains (NAG vibrios) of different origin (Thailand, Algir, Ghana) their cultures, culture filtrates and fractions obtained by precipitation (50–70% ammonium sulphate-10–20 mM zinc acetate), membrane filtration (AMICON PM 10, PM30, XM100A) and gel filtration (Sephadex G-100) were investigated *in vivo* in rabbit ileal loop, in 6-day-old mice in mouse lung oedema test and *in vitro* on CHO and HEp-2 cell cultures and by the enzyme linked immuno sorbent assay (ELISA neutralization). As reference, choleragen (Welcome–Burroghs), *Escherichia coli* LT (purified LT, Dorner) and respective antitoxins were used. NAG vibrios produced at least 3 different heat labile, non-dialysable toxic principles: (1) choleragen like enterotoxin, related immunologically to choleragen and *E. coli* LT; (2) cytotoxin lethal for suckling mice, cytotoxic for CHO and HEp-2 cells and not neutralized by cholera antitoxin, *E. coli* anti-LT and Shiga antitoxin; (3) haemolysin similar though immunologically unrelated to *E. coli* alpha-haemolysin.

ENTEROTOXICITY OF AEROMONAS

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The supernatants of 170 different *Aeromonas* cultures isolated from various sources (food, water, etc.) were studied in a suckling mouse model (SM) enterotoxigenic *Escherichia coli* (EEC) strains B7A (O148 : H28) and B2C (O6 : H16) were used as positive controls. The supernatants caused EEC-like fluid accumulation in the gut in 79.3% for *Aeromonas hydrophila* and 11.8% for *Aeromonas punctata* subsp. *caviae*. Incubation at 56 °C for 10 min eliminated this activity.

SM positive supernatants were haemolytic for sheep erythrocytes in 58.1%; 61.5% of them necrotized the suckling mouse skin and 95.3% were lethal for mice after intraperitoneal injection. The respective data for the SM negative were 12.8%, 36.3% and 45.4%. The results suggest that aeromonads produce enterotoxin-like substances different from haemolysin and necrotoxin, though the latter may also play a role in their enterotoxigenicity.

INCIDENCE IN ENTERITIS OF ENTEROBACTERIACEAE ISOLATES POSSESSING THE HUMAN COLONIZATION FACTOR ANTIGENS

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Human colonization factors CFA I and CFA II were detected by Evans' haemagglutination (HA) typing and by slide agglutination test in H 10407 and PB 176 sera. A total of 462 *Enterobacteriaceae* strains (including 435 *Escherichia coli)* isolated from faecal samples of 250 enteritis patients were examined. Ten strains belonged to HA type I. None of them had CFA I antigen by the slide agglutination method. Six strains fell into HA group II, but had no CFA II by slide agglutination. HA was not observed with 164 strains. Considering weak (+) HA, 36 groups could be differentiated. Strong (++++) HA was more reliable but was shown only by 77 strains. It was striking that 53 strains (11%) showed prompt ++++ mannose resistant (MR) HA, but only with human erythrocytes. Six strains gave MRHA with chicken erythrocytes (HA type IV). Two strains produced marked (++++) MRHA with bovine and guinea pig erythrocytes. Nine strains agglutinated both human and guinea pig erythrocytes. These HA types differed from Evans' HA patterns. The isolates belonged to serogroups common in faeces (O1, O2, O4, O6, O18, O78). Enterotoxigenic isolates possessing CFA I and CFA II colonization factor antigens rarely occur among enteritis patients in Hungary. CFA agar was suitable for developing fimbria antigens of strains with colonization factors. Although the results of + HA are equivocal, and to obtain erythrocytes of 4 different species is complicated, HA typing should be maintained for detecting new colonization factors. Using specific sera to detect CFA I and CFA II antigens by slide agglutination is particularly advantageous.

ASSUMED NEW ADHESION FACTOR OF ESCHERICHIA COLI STRAINS ISOLATED FROM URINE

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More than 200 *Escherichia coli* strains isolated from urine of patients with clinical symptoms were studied for their haemagglutination patterns. The haemagglutination properties of the strains were tested in absence and presence of mannose with human (A, Rh^+), bovine, chicken and guinea pig erythrocytes. About 50% of the strains agglutinated human erythrocytes in the presence of mannose. The haemagglutinating property of representative strain No. 119, was heat labile, did not appear during growth at 18 °C, but was not inhibited by passage in agar. By the technique used for isolating adhesive pili, it was possible to separate the so called "factor 119" of high haemagglutinating capacity. The haemagglutinating property of the strain

No. 119 could be cured by ethidiumbromide–mitomycin C treatment. Immune serum prepared with the wild-type of strain 119 (Ha^+) was adsorbed with the cured (Ha^-) derivative. This serum agglutinated mannose resistant human-Ha^+ *E. coli* strains. The antigenic structure of "factor 119" differed from that of the known adhesion factors (K88, K99, 987, CFI, CFII), "Factor 119" — apart from its antigenicity — has been assumed to be analogous to the known adhesion factors.

ESCHERICHIA COLI ALPHA-HAEMOLYSIN AS ADDITIVE VIRULENCE FACTOR

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The Hly plasmid was cured from a number of *Escherichia coli* strains isolated mainly from oedema disease of pigs. The plasmid was successfully transferred to K-12 and retransferred to the Hly^- derivative of the donor strain. The transferred plasmid reconstructed the original virulence and lent a partial virulence to the K-12 derivative. The virulence of Hly^+ wild types, cured Hly^- and transconjugant Hly^+ derivatives was tested in different animal models. The virulence enhancing effect of the Hly^+ character has been established unanimously. In different mouse models the Hly^+ clones caused consequent haemoglobinuria in mice dying 10–24 hr after challenge. Though the Hly^- derivative persisted for a long period of time in blood, it was less frequent than the Hly^+ one. The higher germ count of Hly^+ clones might be the consequence of a leukocidin-like effect of the alpha-haemolysin. In some cases the conjugation led to the appearance of "non-diffusible" beta-haemolysin production. Preliminary investigations did not support the role in pathogenicity of this type of haemolysin. According to the data, the *E. coli* alpha-haemolysin seems to be an additive factor of virulence.

THE ROLE IN PATHOGENICITY OF PLASMIDS OF ESCHERICHIA COLI STRAINS

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A significant difference in mouse lethality was observed when the animals were injected with Col V^+ and Col V^- strains from patients and healthy carriers. Col V plasmids of two *Escherichia coli* O78 R^+ Col V^+ strains were eliminated and R plasmids of two *E. coli* O78 R^+ Col V^+ strains were partly or entirely eliminated by ethidium bromide. In all cases the LD_{50} of eliminants increased significantly. LD_{50} of the four *E. coli* O78 R^+ Col V^+ strains varied from 4×10^5 to 2.3×10^6 in different periods. After elimination of Col V plasmids of two strains, the LD_{50} increased from 5.7×10^5 to 1.6×10^7. In one strain having lost Cm, Tc and high level Ap resistance, the LD_{50} increased from 5.9×10^5 to 1.1×10^7. In a Tc resistant strain during elimination of Tc resistance plasmid, the LD_{50} increased from 5.4×10^5 to 7×10^6. These findings suggest the presence of a pathogenicity-influencing plasmid that has not yet been demonstrated by genetic studies besides R and Col plasmids in *E. coli* O78 strains.

PATHOGENICITY TESTS WITH YERSINIA ENTEROCOLITICA

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Oral and eye infection with Hungarian isolates of *Yersinia enterocolitica* O3 failed to cause clinical symptoms in different laboratory and domestic animals (e.g. suckling and adult pigs, oral and eye route) and in different zoo animals. A *Y. enterocolitica* strain freshly isolated from a family outbreak produced febrile enterocolitis in a volunteer at 10^9 cells dose; excretion of the organism lasted for 3 weeks. Thirteen years later the same strain, preserved by freeze-drying, 25 other strains isolated freshly from patients and 5 strains from water and food, failed to cause clinical symptoms in the same volunteer.

THE ACTION OF VOLATILE FATTY ACIDS (VFA) IN ESCHERICHIA COLI DIARRHOEA

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The role of VFA in *Escherichia coli* diarrhoea was studied by gas chromatography using weaned rabbits as model animals. The concentration of VFA in the caecum of healthy rabbits ranged 70–130 mM/kg (C_2 87%; C_3 3%; C_4 10%) at pH 5.6–6.4. This VFA-concentration exhibited a marked inhibitory effect on *E. coli* multiplication. The concentration of VFA in the caecum of rabbits with *E. coli* diarrhoea was not considerably different, but the inhibitory effect on *E. coli* ceased as the rising pH value (pH 7.0—7.5) inactivated VFA by forming salts (acetate). Nutrition factors reducing the anti-*E. coli* antivities of VFA in caecum promote the development of *E. coli* diarrhoea.

CALF CRYPTOSPORIDIOSIS IN HUNGARY

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As a result of aetiologic studies on enteric diseases of calves in 1978, out of 103 calves dying from or bled because of diarrhoea, microscopic investigations revealed cryptosporidia in the ileum of 39 calves. Parallel bacteriological cultures detected K99$^+$ *Escherichia coli* known as enteropathogenic for newborn calves in the ileum of 4 cryptosporidium positive calves. Electron microscopy of the intestinal content of further 3 cryptosporidium positive calves detected rotaviruses in one calf. Macroscopic changes, characteristic of intestinal cryptosporidiosis were not revealed. In the ileum of one scouring calf excreting cryptosporidia in its faeces, shortening of villi and hyperplasia of crypts were found by light microscopy. Light and electron microscopy revealed different developmental stages of the extracellular cryptosporidia (3–4 μm in diameter) mainly on the apical surface of the villi, embedded into the brush border. Among calves in Hungary, cryptosporidiosis seems to be a common enteric infection without severe macroscopic changes.

AMINO ACID UTILIZATION AND SYMBIOTIC N_2-FIXING CAPACITY IN RHIZOBIUM MELILOTI

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The wild type *Rhizobium meliloti* 41 establishes an effective N_2-fixing system in symbiosis with the host plant Medicago sativa. These bacteria can grow in liquid medium, containing only one of the amino acids arginine, histidine, lysine, glutamine and asparagine, as sole carbon and nitrogen source. Some ineffective mutants, isolated earlier, pleiotropic defective in tryptone medium induced nitrate reduction, cannot utilize these amino acids as sole carbon and nitrogen source. Based on this phenomenon, mutants in amino acid utilization were isolated and the symbiotic effectiveness were studied. After NTG (nitrosoguanidine) treatment, bacteria were spread onto selective medium containing one single amino acid and small colonies were isolated. Searching for factors related to effectivity in N_2-fixation, the mutants are characterised primarily according to their symbiotic properties (nodulation, effectiveness) and secondly according to their nitrate reduction, auxotrophy, phage and antibiotic sensitivity. It was concluded that a correlation exists between amino acid utilization of free living bacterium and its symbiotic capacity.

ISOLATION AND MAPPING OF RHIZOBIUM MELILOTI MUTANTS AFFECTED IN SYMBIOTIC NITROGEN FIXATION

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Mutants of *Rhizobium meliloti* 41, affected in symbiotic nitrogen fixation, were isolated after nitrosoguanidine or transposition (Tn5 insertion mutation) mutagenesis. Several mutants were unable to nodulate the lucerne host plant (Nod^-), the other mutants did form nodules on lucerne but did not fix nitrogen (Eff^-). To determine the map positions of these mutations, plasmid R68.45 was introduced into the mutants, then these strains were used as donors in conjugation experiments. Seven different double auxotrophic mutants with known map positions were used as recipients. Isolating double prototrophic recombinants, the entire chromosome was transferred. The nitrogen-fixing ability of the recombinants was tested in symbiotic tests. In this way, the map positions of 1 Nod^- and 3 Eff^- mutations were determined.

NITROGENASE ACTIVITY IN GLUTAMINE SYNTHETASE MUTANTS OF RHIZOBIUM MELILOTI

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Involvement of glutamine synthetase (GS) isoenzymes (GSI, GSII) in the regulation of nitrogenase synthesis was studied in mutants which require glutamine for optimal growth. The GS isoenzymes were separated by isoelectric focussing in polyacrylamide disk gels. The three

mutants contained reduced total GS activity. Two of them (ZS231 and GYO516) were similar to Db5, a previous isolate, which has a characteristically low (5–10%) total GS activity low GSI activity. In the same mutants no GSII could be detected. The nitrogenase activity in symbiotic tests was comparable to the wild-type in GY516 and reduced (20%) in the ZS231, unlike in the Db5 mutant in which no nitrogenase could be detected. In the ZS21 mutant there was no GSII detectable, the GSI was slightly reduced and the nitrogenase had an activity similar to that of the wild type. The fact that among GS mutants some had normal nitrogenase activity may be explained in two ways: GS in *Rhizobium* does not regulate directly nitrogenase synthesis, or the biosynthetically inactive enzyme still maintains the regulatory properties of the active GS.

GENETIC MAPPING OF ARG AND LYS MUTATIONS ON THE RHIZOBIUM MELILOTI 41 CHROMOSOME

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Four arginine and two lysine auxotroph mutants were isolated from *Rhizobium meliloti* 41 bacteria by N-methyl-N-nitroso-N-nitroguanidine (1 mg/ml) — 3 hr — kanamycin (200 μg/ml) mutagenesis and selection. Mapping of the two mutations (arg38 and lys15) on the chromosome was performed via plasmid R68.45 conjugation system. EV91 (glyl, leu4, cys46, strR), JA1 (lys15), JA3 (arg38) strains were used as donors, EV172 (phe15, leu4, strR), EV196 (cys46, phe15, strR), AK194 (cys46, glyl, ade4, tyrl, strR), JA1 (lys15), JA3 (arg38) strains as recipients. The results suggest that the leu4 — phe15 — arg38 — cys46 and lys15 — glyl — leu4 sequences are the most likely.

ISOLATION OF AN AMBER SUPPRESSOR STRAIN FROM RHIZOBIUM MELILOTI 41

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As a gift of Professor J. Schell we have two relatives of the RP4 plasmid: RP4Tc^s am bearing an amber mutation in the tetracycline resistance gene; and RP4Tc^s am Su Al Pl beyond the above mutation bears the *Escherichia coli* Su3 allele. This plasmid confers tetracycline resistance (Tc^r) to the host *E. coli* cell N100 Su$^-$ recA$^-$. First, RP4Tc^s am Su Al Pl plasmid was transferred from *E. coli* to *Rhizobium meliloti*. The *R. meliloti* transconjugants were kanamycin resistant (Km^r) but tetracycline sensitive (Tc^s), indicating that the Su3 allele of *E. coli* was not active in *Rhizobium*. Fifty out of 120 Tc^r isolated clones of *Rhizobium* were investigated further for (i) the ability to transfer Tc^r and Km^r; (ii) the effect of plasmid elimination. Eight clones out of the 50 did not transfer Tc^r but did transfer Km^r. After elimination of the plasmid 2 of the 8 (ZP110 and ZP120) clones lost the resistance. When RP4Tc^s am plasmid was introduced back to ZP120 and ZP110, the transconjugants recovered the Tc^r. When a Tra$^-$ am plasmid was introduced into ZP120 the resulting transconjugants recovered the transfer ability (Tra$^+$). The results can be explained if an amber suppressor mutation has arisen on the *Rhizobium* ZP120 chromosome. As a further proof of this suggestion amber mutants of phage 16–3 were isolated on ZP120.

USE OF TI MUTANTS OF PHAGE 16–3 OF RHIZOBIUM MELILOTI FOR DETECTING MUTATIONS IN A SENSITIVE TEST

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Phage 16–3 forms turbid plaques at 28 °C and 36 °C, 16-3 ti mutants form turbid plaques at 28 °C and clear ones at 36 °C. The ti mutations map into the C gene of the phage, which codes for the repressor protein. The repressor is heat sensitive in the ti mutants. A sensitive technique has been worked out to detect few (1–500) turbid plaque forming phages over the background of a large number (10^6 or more) clear plaque formers. Using ti mutants, the forward mutations can be detected as clear plaques at 28 °C, while the back mutations at the ti site as turbid plaques at 36 °C. The method is simple and fast: a mutagen treated population of phages is plated and part of the samples is incubated at 28 °C, the other part at 36 °C for 24 hr. The experiment can be carried out with free phage suspension as well as with phage infected bacteria, depending on the nature of the mutagenic agent (i.e. acts on replicating on non-replicating DNA). With the help of 3 intracistronic double mutants, the detection of small deletions has been worked out.

ANALYSIS OF HETEROZYGOTES OF RHIZOBIUM MELILOTI PHAGE 16–3

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If a clear plaque forming mutant (c-mutation in the C cistron) was crossed with a turbid plaque former (c^+), the progeny formed c^+/c mottled plaques in a few per cent. According to physical and genetic data this is possible only if these phages bear mismatch in the C cistron. When clear plaque mutant bearing two mutations (Sp4–ti4) in the C cistron was one of the parents, the repair of $ti4/ti4^+$ mismatch (i.e. single site) is reflected by the C/ti4 mottled plaques. Only 6/79 of mottled plaques were C/ti4 type, which indicates that mismatch repair of *Rhizobium meliloti* is ineffective on the phage chromosome. When clear plaque forming point and various deletion mutants (the deleted segment varied from <50 to 7200 base pairs) were used as one parent, mottled plaques were observed only in those crosses, where the point mutant or the smallest deletion mutants were used. The result can be explained in two ways. Either the repair of *R. meliloti* is fairly effective in eliminatiag large single stranded loops, or extensive deletions inhibit the lateral migration of the crossed strands in recombination intermediaries decreasing the chance to form a mismatch which can be identified as clear/turbid mottled plaque. The above explanation is compatible with the suggestions of the Hotchkiss or Meselson–Radding recombination models. The survival of mismatches (indicated by clear/turbid mottled plaques when point mutants crossed with wild type) and small single stranded loops (indicated by mottled plaques in wild type $\times$ small deletion mutant crosses) is in agreement with unpublished mapping results. It is suggested that the level of some nuclease function (RecB, C?) is low in rhizobia.

THE BACTERIOPHAGE OF RHIZOBIUM MELILOTI PERFORMING GENERALIZED TRANSDUCTION

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By the use of the temperate phage 11 of *Rhizobium meliloti* 41 capable of generalized transduction, a genetic information transfer system was worked out for the fine mapping of the bacterial chromosome. The generalized transduction was proved by the transfer of various markers from distant loci on the bacterial chromosome. For further evidence, the bacteriophage was grown in antibiotic resistant donor bacteria with bromodeoxyuridine labelled with heavy DNA. From the phage lysate the infective and transducing particles were separated by CsCl equilibrium density gradient centrifugation according to the distribution of phage titre and transducing activity among the fractions. The higher density of the transducing particles showed that bacterial DNA was packaged in them, instead of their own genom. The transducing phage 11 was compared with other *R. meliloti* phages. It was found to be closely related to temperate phage 16-3. They did not differ in serology, size, shape, density, DNA composition and molecular weight, but phage 11 did not form stable lysogenes and its burst size was five times larger. Among the progeny of phage 11, the frequency of virulent mutants is remarkably high. These are different in their superinfection specificity and we have shown by crosses that the mutation sites are in various loci of the regulation of temperate character. The transducing activity of the mutants is also changed, the explanation of which may be indirect.

MUTANTS ALTERED IN GENETIC RECOMBINATION OF PHAGE 16–3 OF RHIZOBIUM MELILOTI

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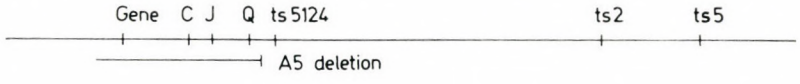
In the course of the analysis and resynthesis of virulent mutants of *Rhizobium meliloti* phage 16–3 we have concluded that there were two genes between cistron C and mutation ts5124 (the nearest early cistron), responsible for the genetic recombination of the phage. The genes were termed J and Q: J^+Q^+, wild type, recombination was normal, showing a direct correlation with the distance. The J^-Q^+ recombination was region specific (around ts5124 point), where the increase in recombination was 20-fold; J^+Q^- recombination was severely inhibited on one arm of the chromosome, while normal in the other direction; J^-Q^-, phage recombination, was inhibited 20–100-fold along the chromosome. With three parental crosses, where one parent was the recombination donor, the effect was followed by the recombination of the two other parents. It was shown that the effect of J^- was trans-dominant, i.e. J^- allele concerned with a structural gene of a protein involved in recombination process. The map positions of J and Q deduced from multifactorial crosses are



COMPARISON OF LINKAGE MAPS OF RHIZOBIUM STRAINS

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The first linkage maps of nitrogen-fixing *Rhizobium* strains were constructed recently in 3 laboratories for 3 different strains: *R. meliloti* 41, *R. meliloti* 2011 and *R. leguminosarum* 300. In this study the arrangement of markers on the 3 linkage maps were compared. Using a map function described previously, linkage data of the 3 maps were converted into additive map distance values. The arrangement of markers was found to show great similarities on the three maps. In chromosome mobilisation experiments between *R. meliloti* 41 and *R. meliloti* 2011 the recombination frequencies and linkage values along the entire chromosome, were about the same as in crosses between derivatives of *R. meliloti* 41, whereas between *R. meliloti* 41 and *R. leguminosarum* 300 recombinants appeared only at a very low frequency. R-prime plasmids carrying segments of the *R. meliloti* 2011 chromosome and complementing auxotrophic mutants of *R. leguminosarum* 300, were tested for complementation of mutants of *R. meliloti* 41. It was found that these R-primes did complement auxotrophic markers located at the same position on the *R. meliloti* and *R. leguminosarum* maps, indicating the functional identity of these markers. Furthermore, it was shown that starting from the site of homology these R-primes promoted polarised chromosome transfer in *R. meliloti* 41.

PLASMID ELIMINATION FROM RHIZOBIUM MELILOTI AT ELEVATED TEMPERATURE

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For the elimination of plasmids from *Rhizobium meliloti*, a heat-treatment procedure was developed. In bacterial cultures grown at 39.5–40.0 °C, the elimination of the conjugative plasmids R68.45, RP4, pJB4JI and their derivatives were demonstrated. The curing of plasmids was followed by testing the loss of plasmid markers. In several cured isolates the lack of plasmid DNA was demonstrated by agarose gel electrophoresis of the bacterium lysates. This curing procedure is useful in strain construction: recombinants obtained after chromosome mobilization with R68.45 can be cured of plasmids. It was found earlier that *R. meliloti* 41 harbours as large plasmid, and certain genes coding for symbiotic nitrogen fixation may be located on this plasmid. The wild type *R. meliloti* 41 was subjected to heat-treatment and individual colonies were tested for nodulation on lucerne host plant. It was found that 20–60% of the cells lost the ability to nodulate lucerne (Nod$^-$). On the other hand, 8 Nod$^-$ clones tested so far did contain the large plasmid. Further experiments are in progress to determine whether the Nod$^-$ phenotype is due to a deletion on the large plasmid or the elevated temperature has a selective advantage for the Nod$^-$ mutants.

CONSTRUCTION OF RECOMBINANT DNA CLONES DERIVED FROM RHIZOBIUM MELILOTI 41

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Our aim was at cloning genes of the symbiotic nitrogen-fixing *Rhizobium meliloti* on the wide host-range plasmid RP4. *R. meliloti* DNA was subjected to limited digestion with enzyme HindIII and then ligated into the HindIII site of RP4 DNA. Recombinant plasmid molecules were recovered after transformation into *Escherichia coli* and recognized by the loss of the kanamycin resistance marker of RP4. Recombinant RP4 plasmids were transferred into *R. meliloti* 41 by conjugation. Plasmid RP4 has no chromosome mobilising ability in *R. meliloti* 41. Thirty-one recombinant RP4 clones were tested for chromosome mobilisation; 4 clones promoted polarized transfer from a single chromosomal site. These sites probably correspond to the regions inserted into recombinant RP4. The technique is suitable for the isolation of any chromosomal region and for its localisation on the genetic map. Furthermore, the use of a set of such recombinant RP4 plasmids facilitates the mapping of new mutations in *R. meliloti* 41.

THE EFFECT OF CHLORPROMAZINE ON PLASMID REPLICATION, PLASMID-TRANSFER AND SEX-PILI OF ESCHERICHIA COLI

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Chlorpromazine (Cpz) caused curing of F'lac and F-like R-plasmids with a frequency of 70% and 15–25%, whilst the efficiency of curing of R-factors belonging to N and I compatibility groups was lower (2–5%). The frequency of transfer of R-144 drd and R-100 drd was reduced markedly (2–3 exponents) by chlorpromazine at a concentration of 50–100 μg/ml. As demonstrated by electron microscopy and plaque assay, chlorpromazine reduced the adsorption rate of male specific RNA phages MS-2 to F-pili.

PHAGE-SPECIFIC DNA SYNTHESIS IN MYCOBACTERIUM PHLEI

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The mechanism of phage-specific DNA synthesis after phage infection was studied in [^{3}H]-thymidine-labelled *Mycobacterium phlei*. (i) Phage-specific DNA synthesis begins at about 30 min after infection and reaches its maximum at 90–100 min. (ii) A large molecular weight phage-specific DNA intermediate could be detected in lysozyme-sodium dodecylsulphate (SDS) lysates after sucrose gradient centrifugation. This phage-specific DNA proved to be sensitive to SDS and was cleaved to smaller pieces at room temperature.

THERMOSENSITIVE FACTOR INHIBITING BUTYRICUM PHAGE PROPAGATION

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Plating efficiency of butyricum phage proved to be 10^{-4}–10^{-3} on *Mycobacterium smegmatis* Rabinowitz cells in logarithmic phase. This increased to 10^{-2}–10^{-1} when before infection the cells were heated for 2 hr at 50–55 °C. Heating of the cells in buffer or in medium did not influence the increase. On incubating the treated cells at 37 °C, the plating efficiency decreased. The decrease was inhibited by chloramphenicol (10 μg/ml) and nalidixic acid (150 μg/ml) but not by mitomycin C (0.05 μg/ml). There was no change in the plating efficiency after heat-treatment if the cells were incubated in Tris buffer. These data suggest that butyricum phage propagation is inhibited by a heat labile factor in *M. smegmatis* Rabinowitz cells.

A NEW TYPE OF HAEMIN-REQUIRING BACILLUS SUBTILIS MUTANT

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A new type of haemin-requiring mutant was isolated by streptomycin selection from *Bacillus subtilis* strain 168 *trp*C2. It accumulates large amounts of uroporphyrin I and coproporphyrin I extracellularly and intracellularly. It does not contain protoporphyrin in detectable quantity. A cell-free extract converted delta-amonilaevulinic acid to uroporphyrin I and coproporphyrin I. When incubated together with an extract of an uroporphyrinogen I synthase deficient strain, protoporphyrin was formed. The mutant is uroporphyrinogen III cosynthase deficient. The *hem*D gene determining the enzyme was mapped by two- and three-factor transduction crosses. This gene is linked to the *hem*A, *hem*B and *hem*C genes, and is distant from the other *hem* genes mapped so far, which are situated on the other replication arm of the *B. subtilis* chromosome. The mutant appears suitable for porphobilinogen → uroporphyrinogen III transformation and for the study of the branching of the porphyrin-corrinoid synthesis. As regards porphyrin synthesis, the behaviour of the described mutant is analogous to what is observed in human congenital porphyria.

GENETIC AND BIOCHEMICAL ANALYSIS OF AMPICILLIN RESISTANCE

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The ampicillin resistance plasmid was transferred to plasmid-free strains by conjugation performed in several steps. The transferred extrachromosomal factors were isolated from the exconjugants and analysed by gel electrophoresis.

BIOSYNTHESIS OF THE CELL WALL OF SHIGELLA SONNEI. I. ISOLATION AND CHARACTERIZATION OF DIFFERENT DEFECTIVE MUTANTS

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Shigella sonnei treated with UV irradiation and alkylating agents and old cultures of the organism yielded a great number of induced and spontaneous mutants. With R-specific phages, the mutants were classified into 15 main groups. From representatives of the main groups the lipopolysaccharide antigen was isolated and analysed for chemical components. The mutants were classified into 6 chemotypes characterized by different degrees of lipopolysaccharide biosynthesis ranging from complete S antigen to polysaccharide-free absolute R antigen.

BIOSYNTHESIS OF THE CELL WALL OF SHIGELLA SONNEI. II. NUCLEOTIDE SUGARS OF R MUTANTS AND THEIR ROLE IN LIPOPOLYSACCHARIDE ANTIGEN SYNTHESIS

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Bacteria with different degrees of R mutation lack certain enzymes of lipopolysaccharide antigen synthesis. Transferase deficient strains are unable to incorporate activated sugars into the polysaccharide part of the antigen and, accordingly, the concentration of nucleotide sugars increases in their cells. By chromatography of different R chemotype mutants, new nucleotides containing activated D-glycero-D-mannoheptose and 4-animopentose were isolated and analysed for chemical structure. The heptose nucleotide could be characterized as adenosine diphosphate-D-glycero-D-mannoheptose.

CHARACTERIZATION OF THE PLASMIDS OF EPIDEMIC SHIGELLA SONNEI STRAINS

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Out of the 2596 *Shigella sonnei* strains phage typed in 1978, 23.1% were homogeneous according to phage type, colicin type, and antibiotic resistance (phage type 2, colicin type 12, streptomycin and sulphonamide resistant). Out of *S. sonnei* strains isolated from different outbreaks, 60.7% belonged to this type. From five different outbreaks 42 strains were examined. The MIC values to streptomycin were 125–60 μg/ml. The R plasmids were fi^- and did not cause phage restriction. The strains were sensitive to 14 colicins (A, B, E1, E2, E3, G, H, Ia, Ib, K,

M, S, V, X) and were lysogenic. The lytic spectra of the carried temperate phages were identical. Comparibility properties of R plasmids were tested in one strain from each of the five outbreaks. Plasmids were related to Iα, and H. Identity of the plasmids of all strains was verified by gel electrophoresis.

THE EFFECT OF PLASMIDS CODING FOR ANTIBIOTIC RESISTANCE ON GROWTH OF STAPHYLOCOCCUS AUREUS IN VITRO

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The carriage of plasmids coding for penicillin, tetracycline, chloramphenicol and macrolide + penicillin resistance has no influence on the growth kinetics of strains in media containing no antibacterial drugs. The transducing phage is responsible for the reduction of the generation time of the recombinant deficient mutant after acquisition of the plasmid coding for penicillin resistance. The growth rates of penicillin plasmid positive and plasmid negative strains are equal in media containing antibacterial drugs, except for erythromycin and ephedrine. In erythromycin the lag period and the first part of the exponential growth of the plasmid negative variant are reduced while in ephedrine the plasmid positive cells exhibit much shorter exponential period. The proportion of penicillin resistant plasmid positive: negative cells during incubation in mixed culture changes from 50 : 50 to about 80–90 : 20–10%.

GENETIC EXAMINATION OF STAPHYLOCOCCUS AUREUS STRAINS RESISTANT TO PENICILLIN AND MACROLIDES

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Staphylococcus aureus JL47/32, JL47/A2 and JL47/C2 — transductants of *S. aureus* 8325 carrying macrolide (Mc) and penicillin (Pe) plasmids from different clinical wild type strains isolated from the outbreak — proved to be sensitive to spiramycin (Sp) and lincomycin (Lcm). In contrast, *S. aureus* RN11 — transductant of *S. aureus* 8325 carrying the Mc Pe plasmid pI258 found in Japan 16 years ago — showed a high degree of Sp and Lcm resistance (MIC > 800 μg/ml). Beside this phenotypic difference between the pI258 and Mc Pe plasmids found in Hungary, another difference was revealed examining the inducer role of erythromycin (Em) in the macrolide resistance of Hungarian strains. Clinical wild type PM2104, one of its Mc Pe resistant transductant JL73/23, and another wild type strain PM1669 showed Em inducible Em resistance in contrast with the constitutive macrolide resistance of *S. aureus* RN11. Results of physico-chemical studies of cell DNA from the Hungarian clinical wild type strains PM2104 and PM1007, from their eliminants (lacking the Mc Pe determinant) and from their Mc Pe resistant transductants confirmed the former genetic results for extrachromosomal localization of Mc Pe determinant in the cells.

INCIDENCE OF STAPHYLOCOCCUS AUREUS AND R PLASMID CARRYING ESCHERICHIA COLI IN WORKERS EMPLOYED IN ANTIBIOTIC PRODUCTION

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Healthy individuals employed in an antibiotic production plant and college students were compared for incidence of *Staphylococcus aureus* in their nose and throat and of *Escherichia coli* in their faeces. Nine out of 24 workers carried mostly tetracycline resistant *S. aureus of* different phage patterns. Nineteen out of 24 students carried staphylococci with their majority resistant to penicillin, tetracycline and chloramphenicol. Fourteen workers excreted tetracycline resistant *E. coli;* one strain carried R plasmid. From 14 students, tetracycline resistant *E. coli* strains were isolated: 2 strains were ampicillin, 1 chloramphenicol resistant; from 4 strains tetracycline, from 1 ampicillin resistance carrying R plasmid was shown by transfer to *E. coli* K-14 Hfr H lac^+ and lac^- recipient. The results failed to confirm the assumption that, as compared to normal populations, workers employed in antibiotic production carry bacteria more resistant to antibiotics.

RAPID METHOD FOR TESTING ANTIBIOTIC SENSITIVITY

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Using the principle of the Autobac system, a simple method has been elaborated for the determination of antibiotic sensitivity patterns in combination with rapid identification. The minimum inhibitory concentrations for 12 antibiotics are determined on Takátsy plates at 0.2 ml volumes in 4–5 hr. Bactericidal concentrations are tested by replica technique read after incubation overnight.

RAPID DETERMINATION OF ESCHERICHIA COLI O ANTIGENS AND ITS USE IN CLINICAL PRACTICE

F. Baron

Public Health Station, Kaposvár

The bacterial suspension of a 16–20 hr agar culture is heated at 100 °C for 10 min and pipetted into wells of plastic plate containing the antisera. Readings are made with the naked eye after incubation at 50 °C for 30 min. The method was used for serogrouping of strains isolated from enteritis and non-faecal samples. In urogenital infection of females, comparison of the serogroup of strains isolated from the urine, vagina and faeces was useful for distinguishing contaminants from the pathogenic bacteria.

RAPID ASSAY OF ANTIBIOTICS AND CHEMOTHERAPEUTICS IN BIOLOGICAL MATERIALS

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Public Health Station, Szekszárd, Ministry of Health, Budapest and Public Health Station, Zalaegerszeg

The medium containing the test organism is added to dilutions of the sample (serum, plasma, urine and other body fluids) on Microtitrator plates. Minimum inhibitory concentrations are read after 3 hr incubation.

DETECTION OF INHIBITORY SUBSTANCES ACTING ON THE MOTILITY OF BACTERIA

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Public Health Station, Szekszárd

Intensely motile bacteria inoculated into the centre of a filter paper strip immersed in broth move peripherically forming a circle proportional in size to the time of incubation. The area of swarming is made visible with a suitable indicator. Salmonellae form in 3–4 hr a circle 20 mm in diameter, then a red peripheral ring. As motility is inhibited by antibiotics, chemotherapeutics, disinfectants, colicins, toxins and certain enzymes, the test may be used for rapid detection of these agents.

A SIMPLE METHOD FOR ANAEROBIC CULTIVATION

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A small sterile glass or plastic dish placed near to the margin of a sterile Petri dish is poured round with freshly prepared anaerobic blood agar medium. The medium is allowed to solidify and inoculated with the sample Then a K_2CO_3-preimpregnated and dried filter paper coil 11 mm in height is placed into the small dish. The filter paper should be rough to form perpendicular gaps between the layers. Two ml pyrogallic acid (20%) are poured from a tilted glass on the paper coil, then the dish is covered with the bottom of a Petri dish of corresponding size. The dishes are fastened together airtight with adhesive strip.

NEW SELECTIVE MEDIUM FOR THE ISOLATION OF HAEMOPHILUS SPECIES

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Haemophilus species are relatively resistant, whereas Gram-positive bacteria in the upper respiratory mucosa are usually sensitive to lincomycin. The lincomycin sensitivity of 100 *Haemophilus* strains of 3 species *(H. influenzae, H. parainfluenzae* and *H. parahaemolyticus)*

was examined by disk and plate dilution methods. For the isolation of haemophilus, chocolate agar containing 5 μg per ml of lincomycin was adequate as confirmed by the examination of colony counts, colony morphology and microscopic picture of the isolates. Of 220 bacterial strains of the normal pharyngeal flora, the majority of Gram-positive strains failed to form colonies on the selective plates. The selective culture medium yielded good results when testing 50 throat swabs.

CHARACTERIZATION OF BACTERIA ISOLATED FROM THE URINE OF PREGNANT WOMEN

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Parallel examination of about 4000 pregnant women with the classical dilution method and with Uricult technique revealed significant bacteriuria by both methods in 10% of the patients. Two-third of the patients excreted *Escherichia coli* belonging in their majority to serogroups O4, O6, O7, O18, O21, O26 and O75. The strains produced colicin X, V, B, E and I. B group haemolytic streptococci were isolated in 2% only with the classical method.

PHAGE TYPING OF LISTERIA MONOCYTOGENES

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Twenty-two *Listeria monocytogenes* phages were isolated from lysogenic strains with or without induction. The phages were selected according to lytic spectrum, trypaflavine and UV sensitivity and hydroxylamine inactivation. Fifteen of the phages were isolated from serotype 1/2, five from 4f, and one each from 4g and 4b strains. By phage typing, 300 serologically characterized strains were classified into 31 phage types. The new phages supplement the phage set for listeria consisting previously of 15, allowing the differentiation of 21 phage types. By neutralization test, the typing phages were classified into 6 groups at least.

COMPARATIVE EXAMINATIONS WITH TNSA MEDIUM CONTAINING POLYMYXIN

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Faecal samples of 75 cattle and of 39 persons and vaginal discharge of 76 women were examined for *Listeria monocytogenes*. After enrichment in Holman's cooked meat medium containing 5% sheep blood, at 277 K for some weeks, the cultures were streaked on TNSA (trypa-

flavine, 15 μg/ml; nalidixic acid, 40 μg/ml; 5% serum) agar plates with and without polymyxin B (3 μg/ml). From the plates containing polymyxin B more *Listeria* strains could be isolated than from the original medium. Fifty-three out of the 75 healthy cattle harboured *Listeria* but only one human carrier was found. Similar results were obtained when agar plates with trypaflavine (15 μg/ml), oxolinic acid (40 μg/ml) and 5% serum were used.

ISOLATION OF STAPHYLOCOCCUS AUREUS FROM FOOD SAMPLES CONTAMINATED WITH GRAM-NEGATIVE BACTERIA

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The usual selective medium containing 5% ox blood and 7.5% sodium chloride was insufficient for the isolation of staphylococci from food samples contaminated with certain Gram-negative bacteria. The media described by Giolitti and Cantoni and by Baird and Parker were more effective. The growth of staphylococci on selective media is especially hindered by salt resistant *Enterobacter* strains and by a diffusible substance produced by them. Another reason for the unsatisfactory detection of staphylococci may be the repression during food production of the plasmid responsible for haemolysin activity.

Virology

AETIOLOGICAL ANALYSIS OF MENINGITIS–ENCEPHALITIS EPIDEMIC IN 1978

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An epidemic of acute CNS diseases has been observed from April to October, 1978, with a peak in July. The total number of notified aseptic meningitis and encephalitis cases in 1978 amounted to 826 and 724, respectively. The age-specific morbidity rates were especially high in the age groups under 6 years. In 47 cases the encephalitis was fatal, and in 44 cases paralytic symptoms were observed. Specimens received from 510 reported and 279 non-reported meningitis as well as from 457 reported and 326 non-reported encephalitis cases were investigated virologically. The aetiology could be clarified in 47% of the cases. Enterovirus 71 (E71) could be incriminated for 20%, tick-borne encephalitis (TBE) virus for 18.6%, coxsackievirus B4 and B5 types for 1.8%, LCM virus for 1.3%, mumps virus for 3.9%, herpes simplex virus for 0.8% and other viruses for 0.4% of the cases. E71 was the main aetiologic agent of CNS diseases occurred among children, whilst the TBE virus of those among adults. The former infections were common all over the country in contrast to the latter which were mostly confined to areas well-known to be natural foci of TBE virus. E71 proved to be responsible for 13 cases with poliomyelitis-like paralytic symptoms and for 8 fatal encephalitis, all among young children.

ISOLATION AND IDENTIFICATION OF ENTEROVIRUS TYPE 71 STRAINS

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Virus isolations were attempted in tissue cultures from 1952 specimens obtained from 686 patients (352 with encephalitis, 330 with meningitis and 4 with hand-foot and mouth disease) during the meningitis–encephalitis epidemic in 1978. Stool samples were available from 454, throat swabs from 369, CSF samples from 401 and p.m. CNS samples from 13 patients. Materials from 620 patients were tested both in Vero and in primary rhesus monkey kidney (RMK) cells, from 18 patients in Vero cells and from 48 patients in RMK cells exclusively. Some specimens were also tested in HEp-2 cells, in primary human embryonic fibroblasts, or in alternating cell systems. Enterovirus type 71 (E71) strains could be isolated from 47 stool samples, 5 throat swabs and 6 CNS samples, originating from 44 patients (34 with encephalitis, 8 with meningitis and 2 with hand-foot and mouth disease). Virus isolations were successful exclusively when Vero cell cultures were used either alone or in combination with RMK cell cultures. Even in this system 3 to 6 blind passages were needed for the isolation of strains. Some difficulties were encountered with the identification of isolates; they had to be pretreated with ether in order to obtain an appropriate neutralization with E71 type sera. Attempts failed to isolate virus in suckling mice from 90 materials of 52 patients. Nevertheless, 5 strains isolated in cell cultures proved to be pathogenic to suckling mice causing alterations characteristic of coxsackie A viruses. In the course of these investigations coxsackie B4 strains were isolated from 8, coxsackie B5 strains from 26, adenovirus type 5 strains from 2 and adenovirus type 7 strains from 1 patient. The majority of coxsackie B strains could easily be isolated both in Vero and in RMK cell cultures.

SEROLOGICAL DETECTION OF ENTEROVIRUS TYPE 71 INFECTIONS

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In connection with a meningitis–encephalitis epidemic in 1978, altogether 1669 serum samples taken from 1050 patients were tested for the presence of neutralizing antibodies to enterovirus type 71 (E71); 845 samples were received from 494 patients with encephalitis, 819 samples from 553 patients with meningitis and 5 samples from 3 patients with hand-foot and mouth disease. As test method, single-radial-plaque-neutralization has been used throughout. Only a single serum sample could be tested from 632 patients whilst two or more samples from 418 patients; 307 patients in the former and 286 in the latter group proved to have antibodies to E71. In 46 cases a significant titre increase and in 6 cases a significant titre decrease could be demonstrated. In order to investigate the possible role of E71 in cases when only a single serum was available and when no significant titre changes could be found, attempts were made to demonstrate E71 specific IgM antibodies in 684 samples of 512 patients. The IgM antibodies were separated by ion-exchange batch technique using QAE Sephadex A-25 gel, and tested by the neutralization method mentioned above. Examination of sera from 74 patients whose E71 infection had already been verified by virus isolation and/or by a significant titre change showed that specific IgM antibodies are demonstrable for 1 to 3 months following the onset of illness. IgM

antibodies could be detected in 379 samples of 307 patients. These antibodies were found in 3%, 56% and 68% of patients with unchanged low, medium and high antibody levels, respectively. The aetiological role of E71 could be confirmed by the serological tests altogether in 312 cases (encephalitis, 152; meningitis, 158; hand-foot and mouth disease, 2).

VIRUS-SEROLOGICAL EXAMINATIONS CONNECTED WITH LOCAL MEASLES OUTBREAKS IN HAJDÚ-BIHAR COUNTY IN 1978–1979

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In 3 villages of Hajdú-Bihar county 118 registered measles cases occurred between September, 1978, and April, 1979. With few exceptions the patients were less than 9 years of age, and 86 of them had received measles vaccine in previous years (73%). In order to verify the clinical diagnosis, sera from 95 patients (74 with and 21 without vaccination history) were investigated for measles HI antibodies and those from 76 patients also for the presence of measles specific IgM antibodies. For the detection of IgM antibodies the ion exchange batch separation method was applied. In 42 of 69 paired sera a significant titre rise of measles HI antibodies could be detected. In additional 36 cases the clinical diagnosis was verified by the demonstration of specific IgM antibodies. Altogether 60 vaccinated patients were found, whose serum samples contained measles specific IgM antibodies. On the basis of the epidemiological analysis it has been supposed that, owing to improper storage of the live vaccine, the vaccination of children had remained unsuccessful. This was supported by the fact that during the illness a primary immune response could be observed in spite of the previous vaccination.

ELISA FOR MONITORING CYTOMEGALO- AND RUBELLAVIRUS INFECTIONS DURING PREGNANCY

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Serial serum samples collected from pregnant women were tested for identifying titre movements to cytomegalovirus (CMV) and rubellavirus (RV). So far sera from 3900 pregnant women with normal delivery, and from 125 women with stillbirth were tested. ELISA was performed in Dynatech Microelisa plates sensitized with CMV and RV antigens. As chromogen, o-nitrophenyl phosphate was used. Evaluation was done by a Dynatech Elisa Reader. (i) To CMV 20%, to RV 16% of the women proved to be susceptible. (ii) Infection rate during pregnancy for both CMV and RV was approximately 1%. (iii) Regarding CMV antibodies, the majority of titre rises was detected in the last trimester of pregnancy; this pointed to the endogenous origin of CMV infections. (iv) Altogether 500 pregnant women/year are at risk to deliver babies with malformations due to CMV and RV infections. (v) The rates of serologically identified infections (CMV%/RV%) were for 3900 normal pregnancies, 0.4/0.12; for 185 early births, 3.2/2.6; and for 96 stillbirths, 8.6/1.2.

MODIFICATIONS CONNECTED WITH ANTIGEN PRODUCTION FOR, AND THE METHOD OF, RUBELLA HI TESTS

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QAE Sephadex A25 ion exchange column chromatography proved to be useful for concentration of rubella virus haemagglutinin. By this technique a 4 to 20-fold concentration of the antigen present in Tween–ether treated and untreated media of BHK 21 monolayer cell cultures infected with the "Judith" strain of rubella virus could be attained. This simple method seems suitable to gain appropriate antigen from low-titre tissue culture harvests. Rubella HI titre of 286 sera was compared in 405 tests using pigeon red blood cells and trypsinized human group 0 Rh positive erythrocytes. In 239 comparative tests, identical results were obtained while in 142 test 2-fold and in 24 tests 4-fold or even higher differences were observed. Similar tests for specific IgM class antibodies separated from 88 human sera by ion exchange batch technique gave identical results for 85 samples, but in 3 samples the IgM antibodies were demonstrable exclusively by the use of human erythrocytes.

RAPID DIAGNOSTIC METHODS IN VIROLOGY. I. ELECTRON MICROSCOPE STUDIES OF THE HUMAN REOVIRUS-LIKE AGENTS OF INFANTILE GASTROENTERITIS

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Materials collected from infantile diarrhoeas were treated with freon, concentrated with PEG if necessary, and transferred to Formvar coated grids overcoated by carbon. The preparations were negatively stained with uranyl acetate, and examined in the electron microscope. The whole procedure required 3–4 hr. In the course of an epidemic of infantile diarrhoea, 50 specimens were processed. In the early spring period of the outbreak, virus was detected in 54% of the specimens. Most of the viruses corresponded to *Rotavirus*, the others to *Astrovirus* or *Adenovirus*. In the late spring period, similar viruses were found in 23% of the specimens. The clinical and epidemiological relations of laboratory diagnosis are discussed.

ANTIBODY DETERMINATION IN THE CSF BY IMMUNOFLUORESCENT TECHNIQUE FOR DIAGNOSIS OF VIRAL CNS INFECTIONS

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Tests for virus-specific antibodies in the CSF are used infrequently for the laboratory diagnosis of viral infections of the CNS, mainly because of the modest sensitivity of the generally applied serological methods. In the present study antiviral antibodies of IgG and IgM classes were investigated by fluorescent antibody (FA) technique of increased sensitivity in the

serum and CSF samples taken simultaneously from 20 lymphocytic choriomeningitis (LCM), 12 tick-borne encephalitis (TBE), 10 mumps-meningitis and 12 varicella-zoster (VZ) meningoencephalitis cases. In order to differentiate antibodies present passively in the CSF from those produced within the CNS, in serum and CSF samples, determination of IgG antibody titres to herpes simplex virus (HSV) and to capsid antigen of EB virus (EB-VCA) was carried out by FA technique and the IgG/albumin quotients were estimated by radial immunodiffusion test. The technique proved to be valuable for rapid specific diagnosis of CNS infections caused by LCM, TBE, VZ and mumps viruses, but not for that caused by HSV virus. Moreover, titre changes of IgG antibodies to HSV and EB-VCA in the CSF samples taken serially seem to indicate the dynamics of pathological processes in CNS damage of unknown origin.

VIRAL ANTIGENS IN EPITHELIAL CELLS OF THE UTERINE CERVIX

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To study the interrelationship between latent viruses and host cells, cytological preparations have been investigated by immunofluorescence. Epithelial cells of the cervix from 330 unselected females were examined for the presence of adeno- and herpesvirus antigens. From each female patient at least three scrapings were examined. Hyperimmune sera prepared against adeno- and herpes simplex viruses in rabbits were used. The anti-adeno sera were conjugated with fluorescein isothiocyanate and those against the herpesvirus with rhodamine. Adenovirus and/or herpes simplex virus antigens could be detected in the cervix cells in 16% of women who proved to be negative at colposcopy while 44.8% of those in whom colposcopy showed moderate or extensive alterations. The difference was significant statistically. It was remarkable that the antigens of these viruses with oncogenic capacity were present in the cervical cells of every patient suffering from cervical carcinoma.

ADENO- AND HERPESVIRUS ANTIBODIES IN SERUM OF PATIENTS WITH UROLOGICAL DISORDERS

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Serological studies were undertaken to determine the possible connections between viruses and tumorous growths. Sera from 380 patients with urogenital malignancies and other diseases were tested for antibodies against adeno- and herpes simplex virus, as well as against early — non virion — adenovirus antigens. Results of complement fixation test have shown that antibodies against adenoviruses can be detected less frequently while those against herpesvirus more frequently in patients with urogenital tumour and with prostatic hypertrophy than in patients with other diseases. The difference found in the antibody levels against the non-virion antigens of adenovirus type 12 in the various illnesses was remarkable: patients with prostatic hyper-

tropy and tumours ranked with 54% while urological non-tumorous patients with 18% and internal patients with 4%. The finding may indicate a relationship between these viruses and the malignancies.

DETECTION OF BABOON ENDOGENOUS VIRUS-SPECIFIC ANTIBODIES IN THE PLASMA OF HEALTHY PERSONS AND PATIENTS WITH MYELOID LEUKAEMIA

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Proviral sequences related to baboon endogenous virus (BaEV) can be detected in human cells, and the expression of these sequences is highly increased in leukocytes of patients with myeloid leukaemia, but the incidence of BaEV-specific antibodies among leukaemic patients has not been clarified. In the present study plasma samples of 15 acute (AML), 30 chronic (CML) myeloid leukaemic and 12 praeleukaemic patients, and 30 healthy persons were investigated for the presence of BaEV-specific antibodies by indirect membrane immunofluorescence. Three of 15 untreated patients with AML had antibodies in their plasma and all had a low leukocyte count in peripheral blood. Out of 4 AML patients with prolonged remission after chemotherapy, 3 persons had antibodies. Five CML plasma samples proved to be positive for antibodies. Studies of CML samples suggest that antibodies are detectable only in the quiescent phase of CML within one year from the onset of the disease and when the peripheral leukocyte count is low. Out of 12 praeleukaemic patients, 5 had antibodies. Appearance of acute leukaemia could be observed in 4 patients, who in the praeleukaemic period had proved negative for antibodies.

ISOLATION OF AN APPARENTLY NON-ONCOGENIC MAREK'S DISEASE VIRUS

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Marek's disease virus was regularly demonstrated in SPF chicken flock using egg inoculation technique without any evidence of Marek's disease. Typical cytopathic effect of Marek's disease virus was seen in direct chicken kidney tissue culture prepared from 4–6 weeks old apparently healthy chickens from this flock. Characteristic Cowdry-type "A" intranuclear inclusion bodies and prominent nucleoli were seen in altered cells using acridine orange staining. Examination of ultrathin section of the cells from infected tissue cultures revealed virus particles in the cell nucleus with typical herpes-type morphology. Serological relationship between the isolated virus and a pathogenic strain of Marek's disease virus was demonstrated in agar gel precipitation test. Inoculation of 1 day old genetically susceptible Rhode-Island Red chickens with the isolate caused no Marek's disease during the experimental period, but histologically various degrees of lymphoid cell infiltration could be seen in various organs. The isolate ensured significant protection to susceptible SPF chickens inoculated at one day of age and challenged 3 weeks later by intra-abdominal inoculation of a pathogenic Marek's disease virus.

AETIOLOGY OF AN ACUTE ENTERIC TRANSMISSIBLE GASTROENTERITIS (TGE)-LIKE DISEASE OF SWINE

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In order to clarify the aetiology of an acute enteric disease of swine clinically and epidemiologically resembling TGE, 2 to 10 days old piglets and weaners with acute intestinal symptoms were investigated. The negative results of the direct immunofluorescence (IF) test for the diagnosis of TGE, which is routinely applied on small intestinal scrapings and the negative virus isolation attempts, as well as the absence of neutralizing antibodies to TGE virus in the blood sera, excluded a TGE virus infection. The aetiological role of rotavirus was also excluded by direct IF test and immuno-electron microscopic (IEM) examination of the intestinal contents. Ultrathin sections of the epithelial cells from different segments of the small intestine revealed numerous virus particles with an average diameter of 70 nm. Virus particles occurred within cytoplasmic vacuoles and vesicles surrounded by a unite membrane, and also free in the cytoplasm. Coronavirus-like particles were detected in the negatively stained suspensions of the intestinal contents and scrapings. By IEM, these coronavirus-like particles were found to be antigenically distinct from the 2 known porcine coronaviruses, TGE virus and haemagglutinating encephalomyelitis virus. Up to now, no evidence of viral replication of the coronavirus-like particle has been obtained in different cell culture systems.

IMMUNODIFFUSION TEST FOR THE DIAGNOSIS OF AUJESZKY'S DISEASE

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The laboratory diagnosis of Aujeszky's disease is based on the virus neutralization (VN) test. The high cost of the test called for a more simple method. On the basis of our former experience and data in the literature further development of the micro-immuno diffusion test (MIDT) seemed promissing. Using chemical detergents, virus purification and concentration procedures resulted in an effective precipitation antigen. The threshold limit of sensitivity of this antigen in the precipitation test amounted to 1 : 2 – 1 : 4 of the VN test. The MIDT seems to be suitable for broad screening examinations to establish the infection status of a herd.

INTERFEROGENICITY AND IMMUNOGENICITY OF DIFFERENT RABIES VACCINES

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Interferon (IF) production in CFLP mice was induced by intraperitoneal immunization of Hempt, Vnukovo 32 and PMWI 38 1503-3 M (Mérieux) rabies vaccines. Three hours after immunization the IF titre was higher in the serum than in the brain. The serum IF titre did not

depend on the type of vaccine. In the brain IF was detectable for the longest time (24 hr) when induced by the Mérieux vaccine. The earliest disappearance of IF (< 6 hr) was observed in the brains of mice immunized with the Hempt vaccine. The highest IF titre was induced by the Mérieux vaccine. Ten persons were immunized with the Mérieux vaccine mostly on days 0, 3, 7, 14, 30 and 90. Four hours after inoculation, the IF content in the sera was 40–320 IU/ml. Rabies neutralizing antibody (nAB) was already detectable in the sera on day 14 ($\geq$1.73 IU/ml). On day 90, the nAB content was $\geq$27 IU/ml and there was no decrease till the 110th day. In the serum of a patient the IF and nAB levels were low due to long-term prednisolone treatment.

IMMUNIZATION EXPERIMENTS WITH FORMALIN-INACTIVATED HEPATITIS B SURFACE ANTIGEN

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HB_sAg was extracted and purified from a plasma pool collected from healthy HB_sAg carrier HB_sAg negative blood donors. First a HB_sAg suspension was obtained by gel filtration of Sepharose 6B column and then it was freed from plasma proteins by CM Sephadex C-50 column chromatography and finally it was purified further and concentrated by QAE Sephadex A-25 column chromatography. The purified HB_sAg suspension was divided into two lots. The first lot was inactivated by 0.04% final concentration of formaldehyde at 37 °C for 2 days and at 4 °C for 7 days. Then it was dialysed against PBS. The second lot was incubated in the same way except for formaldehyde treatment. Nineteen guinea pigs were immunized with the two lots each. Twenty μg HB_sAg and 5 mg $Al(OH)_3$ mixture was administered 3-times in intervals of 1 month. Ten days after the last inoculation the animals were bled by heart puncture. For measurement of the immunological response, antibody determinations were carried out by immuno-diffusion (ID), complement fixation (CF) and passive haemagglutination (PHA) techniques. In ID tests the sera were negative for antibodies against human serum proteins but all gave precipitation lines with HB_sAg at 1 : 64 or higher dilution. In CF test sera from 6 guinea pigs immunized with the first and from 5 immunized with the second lot gave a positive reaction in 1 : 3 dilution with human serum proteins. The geometric mean titre of sera in CF test was 1 : 209 in the first and 1 : 148 in the second group, whilst those in the PHA test, 1 : 23 500 and 1 : 56 320, respectively. Thus the technique used for purification of HB_sAg is applicable for appropriate antigen production.

IMMUNOGLOBULIN CHARACTERISTICS OF ANTIBODIES IN THE MILK OF SOWS AFTER ORAL VACCINATION WITH THE "CKp" STRAIN OF TRANSMISSIBLE GASTROENTERITIS (TGE) VIRUS

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The dynamics of the specific TGE virus neutralizing (VN) antibodies were followed up during the lactation period in the sera and milk wheys of sows that had been vaccinated orally or intramuscularly (IM) with the CKp strain of TGE virus or had recovered from TGE. Thereafter, the immunoglobulin classes of the lactogenic TGE antibodies and their specific VN activ-

ity were determined. The average VN antibody titre was generally higher in the colostral whey of both orally vaccinated and naturally infected sows than in their blood serum. Between the 3rd and 7th post-farrowing day, the titre of the milk wheys fell to a level of 2 to 3 $\log_2$ units lower than that of blood sera and then they remained practically constant to the end of the lactation period. In the milk of the IM vaccinated sows, the antibody titres declined markedly from the first day of the lactation period and they disappeared between the 7th to 14th post-farrowing day. In the orally vaccinated sows, the specific VN activity of the lactogenic TGE antibodies was associated primarily with the IgA class like in naturally infected sows. In the IM vaccinated sows, as a result of the parenteral antigenic stimulation, the lactogenic TGE antibodies appeared to be primarily of the IgG class.

SUPPRESSION OF MITOGEN INDUCED PROLIFERATION OF NORMAL SPLEEN CELLS BY SPLEEN CELLS FROM SYNGENEIC CHICKENS INOCULATED WITH HUMAN ADENOVIRUS TYPE 6

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It has been found that a single intravenous injection of human adenovirus caused a transient immunosuppression in chickens. In the present study experiments were undertaken to clarify the mechanism of the suppression. Studies were initiated to detect the presence of suppressive elements possibly responsible for the immunosuppression. Concavalin A (Con A) responses have been measured in spleen cell cultures prepared by mixing normal chicken spleen cells with mitomycin C-treated spleen cells from syngeneic chickens infected with human adenovirus type 6. Spleen cells of uninfected chickens served as controls. It was found that spleen cells of adenovirus-infected chickens significantly depressed the Con A response of normal spleen cells. Marked depression of mitogenic responses was achieved by spleen cells taken from chickens two days after virus infection. The observations suggest that suppressor cells can play a role in the immunosuppression caused by adenoviruses.

EFFECT OF ADENOVIRUS INFECTION ON HUMAN PERIPHERAL LYMPHOCYTES IN VITRO

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It has been observed that the circulating blood lymphocytes of some patients with certain illnesses of unknown aetiology harbour viral antigens. It is not known how the properties of the lymphocytes are influenced by the presence of the viruses. Therefore lymphocytes obtained from the blood of healthy donors (non-virus carriers) were infected with adenovirus type 5 strain and cultured for 5 days. The appearance of viral antigens as well as their local-

ization in the lymphocytes were investigated by immunofluorescence. The capacity of the infected lymphocytes to form E rosettes and to transform into lymphoblasts in the presence of PHA as well as their sialic acid content have been determined. Normal uninfected lymphocytes served as control. The results showed that E rosette formation of the lymphocytes previously inoculated with the virus was appreciably retarded. The effect was not eliminated by PHA or levamisole. When, however, levamisole and PHA were added simultaneously, the ability of virus-infected lymphocytes to form E rosettes became normal and comparable to that of the control. Inoculation of lymphocytes with virus reduced their transformability as well as their sialic acid content.

INTERFERON INDUCTION BY INCOMPLETE PARTICLES OF ADENOVIRUS TYPE 12

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Defective particles were separated from a pool of human adenovirus type 12 suspension by density gradient centrifugation. The defective particles differed from complete virions in infectivity and titre. In contrast, no significant differences were detected in their interferon-inducing capacity. The defective particles even induced T antigen formation. A unique property of incomplete particles was that their interferon-inducing capacity was sensitive to trypsin treatment whereas complete particles were resistant to trypsin. These particles contain viral DNA shorter than the complete viral genome and sequences of the left-hand end of the genome are represented preferentially. It is suggested that this part of the adenovirus genome is sufficient to induce interferon production in chick cells. This was supported by the observation that the interferon inducing ability of adenovirus was more resistant to UV treatment than its T antigen formation.

INTERFERON-INDUCING PROPERTY OF A POLY (A)-CONTAINING RNA FRACTION EXTRACTED FROM ADENOVIRUS INFECTED CHICK EMBRYO FIBROBLASTS. EFFECT OF INTERFERON PRETREATMENT

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Poly(A)-containing RNA preparation was isolated by means of oligo(dT) cellulose chromatography from chick embryo fibroblasts infected with human adenovirus type 12. The RNA preparation induced interferon in cultures of chick embryo fibroblasts but did not do so in mouse L929 cells. RNA extracted from uninduced cells was not active in either cell type. The interferon-inducing RNA could also be isolated from cells pretreated with interferon before induction. Actinomycin D at a concentration of 0.5 μg/ml inhibited interferon production by RNA treated cells. Priming of chick cells with homologous interferon enhanced their interferon production following induction by the poly(A) containing RNA fraction.

INTERFERON PRODUCTION AND LIVER GLYCOGEN CONTENT OF RATS EXPOSED TO HYPOXIA AND HYPOKINESIS

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Wistar rats were exposed to hypoxia and hypokinesis for 30 and 60 days. Hypoxia was attained in a low-pressure chamber and corresponded to that of 7000 m altitude. Rats were immobilized in special plastic boxes. As interferon inducer, tilorone was administered. Serum interferon and liver glycogen were measured at 30 and 60 days, respectively, after exposure of the animals to hypoxia and/or hypokinesis. Levels of circulating interferon induced by tilorone did not change as compared to the controls, while the amount of liver glycogen decreased under hypoxia or hypokinesis and under their combination.

INTERFERON INDUCERS AS RADIOPROTECTIVE AGENTS

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The radioprotective effect of different interferon inducers (tilorone, Acranil, poly I : C, poly G : C, *E. coli* endotoxin) against acute and prolonged ionizing irradiation (X-rays, gamma ^{60}Co rays) was investigated. Inbred BALB/c mice of both sexes weighing 28–30 g (12–14 weeks old) were treated with interferon inducers. Irradiation was carried out at the time of the peak of the blood interferon level. All interferon inducers increased the endogenous spleen colony formation and survival of the irradiated animals. Radioprotective activity of poly I : C was enhanced on combining it with insoluble polyanions. The mechanism of radioprotective activity of exogenous crude interferon preparation and poly I : C was investigated *in vitro* and *in vivo*. Interferon and poly I : C transiently inhibited the proliferation of stem cells presumably in the radioresistant phase of the cell cycle.

MIF ACTIVITY OF HUMAN INTERFERON PREPARATIONS

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The presence of migration inhibitory factor (MIF) in interferon preparations produced by Sendai virus infected human leukocytes was studied. The migration of macrophages was inhibited by crude human leukocyte interferon (HLIF), while purified HLIF had no MIF activity. The physico-chemical and biological properties of MIF and HLIF were studied. (i) MIF lost its biological activity at pH 2, in contrast to the crude interferon. (ii) MIF was less sensitive to trypsin than HLIF. (iii) MIF was active in heterogeneous systems (rabbit,

chick, guinea pig), whereas interferon was species specific. The purified HLIF influenced the yield of MIF induced by concanavalin A. Interferon (100 IU/ml) increased the yield of MIF, thus interferon pretreatment had a priming effect. On the other hand 10^4 IU/ml interferon nhibited the production of MIF.

PRODUCTION, PURIFICATION AND ANTIVIRAL EFFECTIVENESS OF HUMAN LEUKOCYTE INTERFERON PREPARATIONS AGAINST HERPES VIRUSES

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Factors (i. e. storage of blood, efficiency of interferon pretreatment, priming, source, quality and quantity of serum present in the medium, dose of inducer) ensuring optimum yield and kinetics of interferon production in Sendai virus infected human peripheral lymphocytes were studied. The preparations obtained were concentrated and purified by fractionated alcoholic precipitation and their antiviral activity was assayed on tissue cultures alone or in combination with known antiviral substances i. e. IDU and quercetin. The antiviral effect was tested against *Herpesvirus hominis* types 1 and 2, which are relatively resistant to interferon. The interferon preparations given in combination either with IDU or quercetin were more effective than alone. The significance of interferon therapy in combination with other antiviral substances is discussed.

STRUCTURE OF TWO-DIMENSIONAL CRYSTALLINE HEXON ARRAYS ANALYSED BY OPTICAL DIFFRACTION

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During the crystallization process of Ad-1 hexons, two-dimensional crystalline arrays were observed by electron microscopy. The structure of the dense two-dimensional hexon lattice was analysed directly from the electron micrographs and indirectly through its effect on the optical diffraction. The optical diffraction pattern of the crystalline array and of the hexon centre map showed slightly distored hexagonal arrays of spots. First order spectra corresponding to the centre-to-centre particle distance, i. e. the lattice constants were found to be different when measured along the three non-parallel lines connecting the first order reflexion spots being in front of each other. Direct measurement gave similar results. The lattice constants were, however, approximately 10% higher if determined from the three non-parallel hexon rows directly from electron micrographs. The optical diffraction pattern of the negatively stained hexon lattice extends to the second order and that of the hexon centre map to the third order indicating a relatively large short range disorder within the lattice. The differences in the sharpness of the reflexion spots indicate a considerable long range disorder. It can be assumed that optical diffraction is useful for characterizing the structure of the two dimensional hexon arrays.

ADENOVIRUS TYPE 1-SPECIFIC POLYPEPTIDES DETECTABLE IN VIRUS-INFECTED HUMAN AND RABBIT CELLS BY IMMUNOPRECIPITATION

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Human adenovirus type 1-infected HEp-2 cells and RK-13 cells were extracted with different buffers containing salt, detergents and urea. Bovine adenovirus type 2-specific sheep antiserum was used for the identification of hexon antigens. Human adenovirus type-1 virion-specific rabbit antiserum pool, and a rabbit antiserum prepared against human adenovirus type 1-infected primary rabbit kidney cells were used for the identification and precipitation of viral antigens. Immunoelectrophoresis revealed the presence of two polypeptide populations of hexon-specific antigenicity, which were produced by the infected cells but did not assemble into complete virions. The presence of hexon-like polypeptides in all of the prepared cell and chromatin extracts indicate the aspecific adsorption of these polypeptides to different cellular components. There was one structural protein which induced 32 times less antibody if infected rabbit kidney cells were used for immunization in comparison to rabbit sera of animals inoculated with purified virions.

EARLY DNA-BINDING PROTEINS IN CELLS INFECTED BY HERPES SIMPLEX VIRUS

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In a previous work the presence of early complement fixing nuclear antigen has been demonstrated in HEp-2 cells infected with herpes simplex virus (HSV) type 1 and 2. The nuclear antigens could be solubilized from HSV infected cells with Tris–HCl buffered saline (1.7 M NaCl), pH 7.4. Their DNA-binding properties were studied by DNA-cellulose chromatography. The nuclear antigens purified by chromatography could be bound *in vitro* to cell nuclei fixed with acetic acid–methanol treatment and demonstrated by the anti-complement IF technique. The method seems useful for detection of HSV induced nuclear antigens in biopsy materials. Antibodies against these antigens were found in convalescent human sera.

CYTOKININS IN HYDROLYSATES OF RNAs ISOLATED FROM TMV U1 AND U2

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The naturally occurring cytokinins, a group of N^6-substituted adenine derivatives, are present in plants in the free state and as constituents of certain tRNAs. In addition, cytokinins occur adjacent to the anticodon in specific tRNAs of many other organisms, ranging from

mycoplasmas to animals. The presence of cytokinins in a broad spectrum of living systems and the altered hormone levels of virus infected plants stimulated the authors to study whether viral RNAs contain modified nucleotides with cytokinin activity. After extraction with water saturated n-butanol and separation by paper or column chromatography, the hydrolysates of highly purified TMV RNA (TMV U1) were analysed for cytokinins in soybean and tobacco callus bioassays. On the basis of chromatographic similarities it is suggested that the active materials of hydrolysates are the various forms of zeatin and N^6-(Δ^2-isopentenyl)-adenine. Similar results were obtained using hydrolysates of RNA isolated from TMV U2.

IDENTIFICATION OF NEWCASTLE DISEASE VIRUSES BY ONE-DIMENSIONAL PEPTIDE MAPPING OF THE NUCLEOPROTEIN

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Innumerable Newcastle disease virus (NDV) strains have been isolated throughout the world. They are identical antigenically, but three groups can be formed on the basis of virulence. Virulent strains are responsible for severe epidemics. There has been no method to recognize an individual strain and to find out if different outbreaks are caused by different or by one and the same strain. Nucleoproteins of NDV strains were cleaved by *Staphylococcus aureus* V8 enzyme (Cleveland *et al.*) and the peptides were analysed on polyacrylamide gel. Cleveland-peptide patterns of the nucleoproteins of strains isolated from a particular outbreak seem to be identical and distinguishable from those of other isolates. The method promises to allow the identification of strains belonging to the same virulence group but deriving from different epidemics.

PROTEINS AND REPLICATION OF INFECTIOUS BRONCHITIS VIRUS

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Infectious bronchitis virus (IBV) is the prototype of coronaviridae. The following virion polypeptides (VP) were identified on polyacrylamide gel (mol. wt in thousands) : VP 170, 90, 88, 50, 32, 27, 24 and 15. VP 90, 32 and 27 are glycosylated, while VP 50 is phosphorylated. An attempt was made to identify viral polypeptides responsible for the serotype using cross-immunoprecipitin. The virion RNA is a large polynucleotide of positive polarity, thus it was attempted to establish whether coronaviruses followed the replication pattern of poliovirus or togaviruses. Proteins of infected cells were analysed after immunoprecipitation and VP 130, 50, 38 and 32 were identified. In pulse-chase experiments VP 130 and 32 proved to be precursor proteins, while the other two were stable. This finding suggests the presence of subgenomic mRNAs and a togavirus-type replication. RNA isolated from infected cells and fractionated on formamide gradient stimulated the translation of VP 50 and 27. mRNA species were located in 18 to 20S region. Since VP 50 is the main product of 64S virion RNA in the cell-free translation system, the mRNA coding for VP 50 is probably a subgenomic one copied from the 5'-end of the virion RNA.

POLYPEPTIDE PATTERNS OF INFECTIOUS BRONCHITIS VIRUS SEROTYPES

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Infectious bronchitis virus (IBV) serotypes can be classified into two major categories according to the polypeptide pattern of their virions. In 1975, four new serotypes were described in New Zealand and all were characterised by the C polypeptide pattern. The only known Australian serotype also belongs to Pattern C, and this suggests that the N. Z. serotypes are derivatives of the former one. A direct introduction of the Australian strains is excluded. In contrast, in Europe all strains seem to be American in origin.

COMPARISON OF THE RNA STRUCTURES OF INFECTIOUS BRONCHITIS VIRUS BY T_1-OLIGONUCLEOTIDE FINGERPRINT ANALYSIS

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Fingerprints of IBV strains differing in polypeptide pattern (M and C) and in serotype were compared by T_1-oligonucleotide analysis. Fingerprints of the RNA of IBV strains belonging to M and C patterns showed a 20% difference while differences between strains of the same pattern diminished the more related the strains were.

PURIFICATION OF B.K. VIRUS HAEMAGGLUTININ BY AMMONIUM SULPHATE PRECIPITATION AND GEL FILTRATION

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The haemagglutinin present in the cell-free supernatant of B.K. virus-infected cell cultures was concentrated and partially purified by using 50% ammonium sulphate saturation, the concentration that had proved to be the optimum. The recovery rate was 98%. The preparation thus obtained was gel-filtered through Sepharose-4B to separate the haemagglutinin from the bulk of the residual protein. On the average, 70% of the haemagglutinin was recovered from the protein-poor fractions. The purity of the preparations was checked by electron microscopy and immunodiffusion.

EXPRESSION OF ONCORNAVIRAL p30 ANTIGEN IN CULTURES OF LYMPHOCYTES OF BALB/c AND C57B[1] MICE

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Treatment of lymphocytes of BALB/c mice with different inductors is known to result in the production of complete C-type oncornavirus particles. In lymphocytes of C57B1 mice the same inductors rarely elicit virus production. Nevertheless the inducibility of the individual

viral components may be different, as the synthesis of the whole virion is coded by at least 4 autosomal genes. The aim of the present study was to investigate, whether the induction of the p30 antigen coded by the gag gene was independent of the synthesis of the complete virion in lymphocytes of BALB/c and C57Bl mice. The inductors used were IUdR, l-canavanine, cycloheximide, PHA, d-l-p-fluorophenylalanine and con-A. The antigen was detected by indirect cytoplasmic IFA and competitive RIA. After inductor treatment expression of the p30 antigen could be detected in lymphocytes of BALB/c mice and C57Bl mice alike. A difference was only observed in the time of appearance of the antigen: the product of the gag gene could be detected as early as 24–48 hr after the treatment in BALB/c lymphocytes while as late as induction in C57Bl lymphocytes. In both systems cycloheximide and d-l-p-fluorophenylalanine proved to be the most effective inductors.

INTRACELLULAR FLUORESCENCE POLARIZATION OF FLUORESCEIN IN RAT FIBROBLAST CELLS TRANSFORMED BY WILD-TYPE SV40 OR ITS TSA MUTANT

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The intensity of intracellular fluorescence due to the fluorescein released from hydrolysing fluorescein diacetate (FDA), and fluorescence polarization were examined in rat ribroblasts transformed by the wild-type strain $SV_{68}C$, and in those transformed by the tsA mutant of SV40. Fluorescence polarization was significantly more pronounced in the former, while the latter did not differ significantly in fluorescence polarization from the control cells. There was no difference in esterase activity between transformed and control cells. The outflow of fluorescein was less intense from the cells than it was from normal and tsA-transformed cells. The difference may be attributed to alterations in the membrane function of cells transformed by the wild-type virus. The inhibitory effect of NaF was more pronounced in the cell lines transformed by the wild-type virus than in that transformed by the tsA mutant. The difference manifested itself in the inhibition of both FDA hydrolysis and the outflow of hydrolysed fluorescein.

SPECIFICITY AND IMMUNOGENICITY OF THE SURFACE ANTIGENS OF RAUSCHER ERYTHROBLASTS

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Cell membranes were prepared from Rauscher leukaemic erythroblasts of BALB/c mice after iodination. The Triton X-100-solubilized membranes were prepared for electrophoresis by adding the sample directly to a solution of $NaDodSO_4$ containing mercaptoethanol, glycine and Tris–HCl buffer pH 6.8. After electrophoresis most of the $NaDodSO_4$ was removed and the specimens were cut vertically. Gel slices were pulverized and aliquots were removed for assay of the various antigenic specificities. The antigens were detected by inhibition of mem-

brane immunofluorescence. In this reaction monospecific goat immune sera to the p12, p15(E), p30 and gp70 structural components of R-MuLV were used with sera of BALB/c mice immunized with formalin-treated membrane preparation from leukaemic erythroblasts. On the surface of Rauscher erythroblasts a virus-determined cell surface antigen (TSSA) was detected in addition to the gp70, p30, p15(E) and p12 viral proteins. The density of these antigens was very high with the exception of p30. Out of the membrane components, TSSA and gp70 had the strongest immunogenicity in syngeneic mice, while the p30 polypeptide proved to be non-immunogenic.

CHARACTERISTICS OF HUMAN CYTOMEGALOVIRUS- (HCMV) -TRANSFORMED CELL LINES AND ABORTIVELY INFECTED CELLS

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HCMV specific glycoproteins can be demonstrated in the membrane of HCMV-transformed hamster cells (87-TRH-5 and CX-90-B) by infection of these cells with vesicular stomatitis virus (VSV) and the detection of VSV(HCMV) pseudotypes. The 87-TRH-5 cells induce tumours in newborn hamsters. A tumour-derived cell line (TSC-1), however, is oncogenic both in newborn and weaning hamsters. Infection of TSC-1 cells with VSV does not result in the appearance of VSV(HCMV) pseudotypes, which indicates the absence or a low level, of HCMV glycoproteins in the membrane of cells. Both 87-TRH-5 and TSC-1 cells contain C-type particles. Abortive HCMV infection can be established in the normal hamster cell. After superinfection with VSV of the abortively infected cells the cells yielded VSV(HCMV) pseudotypes if the VSV superinfection was carried out at 8–10 days after HCMV infection, but not earlier or later. IgG–Fc binding receptors were detected by immunofluorescence in the membrane of 87-TRH-5, CX-90-B, TSC-1 and HCMV abortively infected hamster cells.

CELL CULTURES TRANSFORMED BY B.K. VIRUS

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A B. K. virus-transformed hamster cell culture (BKT-1, received from Dr. R. A. Mäntyjärvi, Finland) induced tumour in 100% of hamsters inoculated at 2–3 weeks of age with 10^7 cells. The tumour growth was localized, the tumours were lobular and surrounded by a capsule. Histologically, they proved to be fibrosarcomas. In 2 cases (BKT-H1 and BKT-H2) the authors succeeded in cultivating the induced tumours *in vitro*. From fibrosarcomas induced by BKT-H2, a new line was established (BKT-H2-1). Each of the 3 cultures contained the T antigen in 95–100%. V antigen could not be demonstrated in any case. Even fusion with Vero cells failed to induce V antigen. The fluid phase of the cultures showed no haemagglutinating activity.

ACTIVATION OF ADENOVIRUS TYPE 5 CAUSING LATENT INFECTION IN TISSUE CULTURE

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Although the pathomechanism of inapparent viral infections has been studied extensively, the phenomenon has not been clarified. The various adenovirus types able to induce latent infections deserve special attention. They may play a causative role in chronic diseases with recrudescence. Few data are available on the conversion from latency to activation of adenoviruses beyond certain clinical observations. In order to study the activation *in vitro* of latent adenovirus type 5 infection, primary human amnion tissue cultures were inoculated with 0.5–2 CPU virus and subsequently hormones, carcinogens and microbic products were added to the cells in pretested concentration that did not cause cellular alteration. Of the materials applied, only "Pyrago" containing endotoxins of *Escherichia coli* and of other bacteria and fungi induced activation of adenovirus infection. In the tissue cultures infected with subminimal virus dose, "Pyrago" exerted a characteristic CP effect and infective virus could be recovered. Prednisolone failed to induce this conversion in tissue cultures infected with subminimal virus dose, but when the virus level was raised to minimum, the CP effect became expressed and virus replication was enhanced as indicated by control cultures. Diethylstilboestrol and simple carcinogens did not cause changes in tissue cultures infected with subminimal or minimal virus dose. These results suggest that, among other factors, microbes and their products may have a role in the activation of latent adenovirus infection.

IMMUNOELECTRON MICROSCOPY IN VIROLOGICAL DIAGNOSTICS

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Immunoelectron microscopy affords visualization of antigen–antibody interaction on the ultrastructural level. It offers wide possibilities in diagnostic virology, in particular as regards formation of virus-particle aggregates in the presence of specific antibodies. An advantage of this method is its ability to demonstrate the presence of virus agents occurring in natural fluids at concentrations too low to be visualized by direct electron microscopy, or viruses which cannot be cultivated. The quantity of virus particles in, and the morphological characteristics of, a complex depend on the proportion between virus concentration and concentration of antibody. A relative excess of antibody results in small complexes, in which single viruses are covered by antibody envelopes; an equilibrium is characterized by larger complexes where viruses are joined by antibody bridges. As an example of this relationship, polioviruses aggregate in homologous type antiserum. There is also a possibility of using immunoelectron microscopy for serotyping of enteroviruses or for quantifying the antibody response. Some other examples show the diagnosis and typing of adenoviruses which form immunocomplexes in the presence of complement-fixing — i.e. group specific — as well as virus-neutralizing — i.e. type specific — antibodies. Some viruses aggregate spontaneously in natural media: the rotaviruses are a typical example. In most cases they are easily diagnosed by direct electron microscopy in faecal extracts. To enlarge complexes, commercial gammaglobulin may be used

as a source of antibodies; very large complexes are formed after incubation of faecal extracts containing rotaviruses with anti-human anti-IgA. Immunoelectron microscopy represents in addition a useful tool in the diagnosis of hepatitis A virus in faecal extracts; as a source of antibodies can serve hyperimmune chimpanzee sera or convalescent human sera.

IMMEDIATE EARLY HERPESVIRUS ANTIGENS WERE NOT SEEN IN SERIAL SECTIONS OF GANGLIA WITH ESTABLISHED LATENT INFECTION

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Serum to immediate early (IE) and early (E) polypeptides was prepared in rabbits using 2 antigens: an extract from cycloheximide-treated herpes simplex virus type 1 (HSV 1)-infected cells incubated for 4 hr in the presence of actinomycin D and an extract of the same cells incubated for 4 hr in the absence of actinomycin D. ^{14}C aminoacid labelled antigens (pulsed between 1–4, 2–3 and 4–9 hr p.i.) and their precipitates with anti-IE/E and anti-virion sera were analyzed in PAGE. The anti-IE/E serum reacted with at least 2 alpha polypeptides (ICP 110 and ICP 136) and several beta polypeptides (ICP 146, 136, 87, 68, 42 and 32). By anticomplementary immunofluorescence (ACIF) bright granular staining was seen in the nuclei of HSV 1-infected Vero and SIRC cells between 3–8 hr p.i. Latent infection was established in the mouse cervical spinal ganglia (SC 16 strain) and in the rabbit gasserian ganglion (Kupka strain). No positive fluorescence was seen in the serial sections of either ganglia stained by both sera. In sections of the ganglia kept for 7 days in culture and stained at daily intervals by ACIF, both sera revealed positive single neurons and satellite cells from the 2nd day after explantation. Staining of the nuclei of neurons with the anti-IE/E serum might precede by 24 hr the fluorescence brought about by the antivirion serum.

Immunology

ADENOVIRUS-INDUCED IMMUNE RESPONSE IN THE OVINE FETUS

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Ovine fetuses 70–80 days of age, were infected with ovine adenovirus. Nineteen days after the infection virus-neutralizing antibodies were demonstrable in the sera of the infected fetuses and their twin mates at titres of $1:32-1:128$. Electrophoretically, the antibodies proved to belong to subclasses IgG_1 and IgG_2. Injection of the virus into the fetus induced a maternal immune response. Antibody-producing cells were demonstrated in the fetal spleen, but not in the thymus and in lymph nodes. ^{3}HTdR incorporation was demonstrated in 20–22% of spleen cells stimulated *in vitro* with ovine adenovirus antigen.

INCREASED RESISTANCE OF GERMFREE MICE TO DIANHYDRO--DULCITOL (DAD), A LYMPHOTROPIC CYTOSTATIC AGENT

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The sensitivity to DAD of 5-week-old germfree (GF) and specific-pathogen-free (SPF) mice of both sexes (CH3mg, LATI, Gödöllő) was examined. Each mouse was given DAD in a single intraperitoneal injection. The GF state of mice and environment was maintained in VELAZ P-01 isolators for 21 days after treatment. The respective mortality rates following doses of 12, 15 and 18 mg/kg were 10%, 50% and 70% (SPF mice) and 0%, 15% and 30% (GF mice). SPF and GF mice died 5–8 days and 7–13 days, respectively, after treatment. Thus, as judged by their survival, GF mice showed an increased resistance to DAD. Lymphoid atrophy was more severe in dead GF mice (relative spleen weight, 0.26) than in dead SPF mice (relative spleen weight, 0.4). The results show that the microbial flora might increase drug sensitivity during treatment with a lymphotropic cytostatic agent.

THE INFLUENCE OF HEPES BUFFER ON THE MIGRATION OF HUMAN LEUKOCYTES IN THE DIRECT AND INDIRECT MIF EFFECT

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The mechanism of development of cellular immunity was examined in man by the direct and indirect Clausen method; 0.01 M HEPES buffer was used in the culture fluid of lymphocytes. An analysis of the time curves revealed that MIF production lacks any time dependence on the first 3 days of lymphocyte cultivation. If HEPES was removed by a 24-hr dialysis at 5 °C, unmasking of MIF effect became apparent. MIF effect was revealed independently of its migration-inhibiting or migration-stimulating character. (Alternatively, a factor independent of MIF might be produced in the case of increased migration.) When HEPES buffer was used in the medium of lymphocyte cultures, a toxic effect on leukocyte migration was observed. If the dialysis was omitted, MIF production was increased in $^2/_3$ of the cases and inhibited in the rest.

THE HUMAN LETHAL DOSE OF TETANUS TOXIN

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In an accident that occurred many years ago an experimental lot of human pertussis hyperimmune serum was used for therapy. Twently-three children and 2 adults treated with the serum fell ill with tetanus. Ten children died in spite of therapy which involved treatment with very high doses of specific serum injected immediately after the onset of symptoms. Only patients with great body-weight remained alive. According to the laboratory investigation carried out immediately after the accident, the serum contained a very small amount of tetanus toxin but no *Clostridium tetani*. It was stated that the human LD_{75}/body weight of tetanus

toxin was equal to about 10 MLD for 18–20 g mice. The calculated amount of antitoxin required to neutralize *in vitro* the toxin dose lethal for an adult of 70 kg body weight is about 0.05 IU. Considering this ratio, it may be concluded that the use of very great doses of antitoxin for the therapy of tetanus is of no particular benefit. As regards sensitivity to tetanus toxin, man is about 5 times more sensitive than the mouse.

INFLUENCE OF THE PRESERVATION OF ERYTHROCYTES ON THE CORRELATION OF TETANUS ANTITOXIN VALUES MEASURED BY PASSIVE HAEMAGGLUTINATION AND BY BIOLOGICAL TESTS

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The influence of preservation of the reagent on the correlation between the tetanus antitoxin values obtained by means of the passive haemagglutination test and those obtained by the neutralization test was examined in 65 human serum samples. The correlation was found satisfactory if native erythrocytes were used. If, however, the erythrocytes had been preserved with glutaraldehyde or formaldehyde, the respective passive haemagglutination values were twice and 6–8 times as high as the biological values. The deviations also depend on the serum, viz. whether a human serum or a hyperimmune antitoxic serum is used as standard.

QUANTITATIVE DETERMINATION OF TETANUS ANTIBODIES BY THE "ELISA" TECHNIQUE

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Enzyme-linked immunosorbent assay (ELISA) was used to quantitate tetanus antibodies of human origin. The method was optimized in respect of toxoid antigen, incubation period, temperature, and type of microtitration plates. Human hyperimmune anti-tetanus gamma globulin (Human, Budapest) was used for the detection of antibodies. The lowest antibody concentration measured by ELISA was 0.0015 IU/ml. The tetanus antibody content of a panel of donor sera was determined by both the passive mouse protection test and by the ELISA method. A linear positive correlation was found between *in vivo* and ELISA method, the correlation coefficient being 0.78.

DEMONSTRATION OF MYXOMA INFECTION BY "ELISA"

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Enzyme-linked immunosorbent assay (ELISA) was applied to detect myxoma virus and antibodies to the myxoma virus. Serum antibodies were tested by indirect ELISA, using a virus propagated on chicken embryo CAM. The negative and the positive sera were well-

distinguishable by visual evaluation while photometric testing gave qualitative results. The virion content of tissue cultures was determined by the sandwich ELISA technique. The sensitivity and the specificity of the tests (immunodiffusion, complement fixation and virus neutralization) have been studied comparatively.

DETECTION BY IMMUNOOSMOPHORESIS OF STAPHYLOCOCCUS ENTEROTOXIN TYPE B

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A rapid method is described for indicating the presence of a *Staphylococcus aureus* enterotoxin type B by an immunoosmophoretic method. This is applicable only if the negative charge of the toxin molecule is increased to a sufficient degree. This was achieved by formaldehyde (about 0.3%) treatment, for 1 hr in a 45 °C water bath. The test takes 3 hr and the least amount of enterotoxin that can be demonstrated is 0.5–1.0 μg/ml.

MICROBIAL CONTENT OF GASTRIC AND DUODENAL FLUID AND THE IMMUNE RESPONSE

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Serum gastric fluid and A, B and C bile samples were obtained from 143 dermatological patients and IgG, IgA, IgM, C3 and albumin were assayed in the samples. In the gastric fluid routine bacteriological and mycological tests were performed, and the same samples were tested for protozoa. On the basis of the pH of the gastric fluid, the samples were divided into groups. Pathogenic microorganisms were absent in 55 out of 23 hyperacid and 43 normacid samples while in 54 of the 27 hypacid and 50 anacid samples protozoa, bacteria or fungi were demonstrated. When besides hypacidity or anacidity an infection was revealed, determinable amounts of serum protein(s) were present in the gastric fluid and in bile samples A, B an C.

FORECAST OF THE HUMAN EPIDEMIOLOGICAL VALUE OF VACCINES BASED ON LABORATORY STANDARDIZATION TESTS

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While investigating the utilization of vaccine-standardization procedures in human immunization, it was shown that (i) the diphtheria and tetanus immune effect measured in the guinea pig by "one stimulant method" (USP) predicts with 99% probability the human potency of the antigen and also the degree of attainable populational immunity. The smallest laboratory antigenicity which may be expected to induce safe immunity in man was determined in humans. (ii) The active protection test evolved by PRIGGE was found suitable for measuring

"intrinsic antigenicity". The smallest protective value was determined and given in International Immunizing Units (IIU) necessary for inducing safe populational immunity against diphtheria and tetanus.

THE ROLE OF O ANTIGEN IN THE IMMUNITY AGAINST SALMONELLA TYPHI-MURIUM INFECTION

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One to 10 μg of a ribosome preparation obtained from a mouse-virulent *Salmonella typhi-murium* strain was found to protect mice against 1000 LD_{50} of the homologous strain. The fact that the blood serum of the immunized animals contained anti-O agglutinin proved that the preparation was contaminated by O antigen. To clarify the role of the O antigen, the mouse-immunizing capacity of killed vaccines and ribosomal antigens prepared from O^+ and O^- variants was examined. The O^+ vaccines proved more efficient than the O^- ones.

ASSAY OF LEUKOCYTOSIS- (LYMPHOCYTOSIS-) PROMOTING FACTOR FOR ESTIMATION OF THE PROPHYLACTIC VALUE OF PERTUSSIS VACCINE

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Several biological activities of the leukocytosis- (lymphocytosis-) promoting factor (LPF) of *Bordetella pertussis* have been described. LPF induces hypersensitivity to histamine, leukocytosis, lymphocytosis and hypoglycaemia in mice. It shows the characteristics of a biological adjuvant, and increases the production of IgE antibodies. LPF agglutinates red cells and protects mice against *B. pertussis* challenge. High amounts of LPF increase the mouse toxicity of pertussis vaccine. The experimental results were as follows. (i) Freeze-dried reference vaccine was developed for the standardization of LPF assay. (ii) The total leukocyte count can be used for the quantitative determination of LPF of pertussis and DPT vaccines. (iii) A positive correlation was found between the potency and LPF content of vaccines. (iv) Among the inbred mouse strains CBA/Ca, BALB/c, C57B1, DBA/2 and AKR, DBA/2 was the most sensitive and therefore most suitable for the LPF assay. (v) The leukocytosis- and lymphocytosis-promoting effects of LPF decreased in mice preimmunized with pertussis vaccine.

PREPARATION AND LABORATORY TESTING OF FREEZE-DRIED ADSORBED DIPHTHERIA–PERTUSSIS–TETANUS (DPT) VACCINE

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DPT has an important role in the Expanded Programme on Immunization of the WHO aimed at extending immunization against six dangerous infectious childhood diseases. In tropical areas the stability of DPT is limited by the heat sensitivity of the pertussis component.

To increase its stability, different freeze-dried DPT vaccines were developed. The experimental results were as follows. (i) The pertussis component of DPT freeze-dried with 9% sucrose met the requirements after a 6-week storage at 35 °C. After this period the potency decreased. (ii) Nine per cent lactose was also suitable as a freeze-drying menstruum. (iii) The toxoid components proved to be stable in potency over a period of at least 20 weeks at 35 °C. (iv) Adsorbed DPT containing 9% sucrose or lactose was more stable than a DPT of similar composition without sugar stabilizers. (v) To avoid the freeze-drying of aluminium phosphate gel, preparation of freeze-dried DPT–typhoid vaccine is recommended. The potency of DPT–typhoid vaccine was stable at 4 °C over a period of 8 years.

ISOLATION OF SAPONIN FRACTIONS AND INVESTIGATIONS INTO THEIR BIOLOGICAL EFFECTS

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Twenty-six saponin preparations were fractionated by gel filtration. On the basis of their elution profiles, the main components were characterized by means of thin-layer chromatography, colorimetric reactions of selective sensitivity, and quantative haemolysis. (i) Depending on their origin, commercial saponins can be calssified into the 3 main types Quillaja, Gypsophilla and Smilax. (ii) Their common feature is that a haemolysing (H) and a non-haemolysing (NH) fraction can be separated from each. These fractions amount to 80–90% of the samples. Fraction H has a molecular weight of 40 000. Its components form a complex of labile structure under the conditions of separation. (iii) The thin-layer chromatographic composition of the H fraction is characteristic of the main type. All H fractions are rich in aldopentose and contain genins of triterpenoid structure. (iv) The thin-layer chromatographic pattern of the NH fractions is similar in many respects; ketohexose dominates among the carbohydrate components. Their genin structure differs considerably from the triterpenoid skeleton of the H fractions. (v) The immune response to FMD virus is enhanced by the H fractions but not by the NH fractions.

IMMUNOLOGICAL CHARACTERISTICS OF THE FOOT AND MOUTH DISEASE VIRUS

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(i) The replication properties, immunogenecity and stability of some subtypes of O, A, and C types of FMD vaccine strains have been studied. The virus yield of the type-O and C strains was higher than that of type A. As to the immunological properties, types A and C exceeded type O. (ii) The virions synthesized in the substrates and conditions applied retained their immunological properties during the procedures of vaccine production. (iii) The inactivating effects of formaldehyde and ethyleneimine were studied comparatively. The high capsid-

damaging effect of formaldehyde can be moderated by lowering the concentration. Low concentrations of formaldehyde can be made effective by raising the pH over 8.2. The inactivating effect of ethyleneimine is quick and complete, though the capsid is only slightly damaged by the compound. The vaccines, regardless of the vaccine strain and the inactivator used, are stable for at least one year.

VIRULENCE-ENHANCING EFFECT OF IRON ON KLEBSIELLA PNEUMONIAE AND PSEUDOMONAS AERUGINOSA

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The influence of ferric ammonium citrate (FAC) was studied on the virulence of 3 *Klebsiella pneumoniae* and 7 *Pseudomonas aeruginosa* strains belonging to different serotypes and immunotypes. Groups of mice of 20–24 g body weight were inoculated intraperitoneally with different amounts of 6 hr cultures of the strains; other groups of mice received the same inocula and simultaneously 2 mg FAC per mouse. The results of repeated assays have shown that the mouse-virulance of all *K. pneumoniae* and *P. aeruginosa* strains tested was significantly enhanced by FAC.

Mycology

PROTOPLAST FUSION IN CANDIDA TROPICALIS AND THE ANALYSIS OF FUSION PRODUCTS

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Haploid auxotrophic mutants were produced from two wild type strains of *Candida tropicalis* (CBS 644 and 94 T). Protoplast fusion was induced by polyethylene glycol (PEG). The influence on the fusion frequency of the concentration of $CaCl_2$ and PEG as well as of the molecular weight of PEG has been studied. The results confirmed the data of other authors for filamentous fungi in that the concentration of $CaCl_2$ and PEG is critical in the process of protoplast fusion. The resulting nutritional complementation after the fusion process was due to heterokaryon formation and, at a much lower frequency, to spontaneous diploidization. The heterokaryons were unstable and, at segregation of the genetic markers, the proportion of parental types was in some cases equal and in others there was a bias for one of the partners. The cells of the stable prototrophic isolates were more elongated than those of the parental cells, usually also larger and they contained much more DNA per cell. Both parental types could be recovered from these isolates by haploidization with chloral hydrate and low doses of UV light simultaneously. The results indicate that the stable prototrophic isolates were diploid.

INTERSPECIFIC PROTOPLAST FUSION BETWEEN CANDIDA ALBICANS AND CANDIDA TROPICALIS

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Fusion of *Candida albicans* and *Candida tropicalis* protoplasts was implemented using auxotrophic mutants of these strains. Protoplast formation was induced by snail enzyme in 0.6 M KCl, the fusion by 30% polyethylene glycol (PEG, mol wt 4000) in the presence of Ca^{++}. During selective regenaration of the fusion products, viable and multiplying prototrophic hybrids were obtained. The complementation frequency was $2\times$ to 5×10^{-6} if the calculation was based upon the number of colonies growing on minimal medium divided by the number of those growing on complete medium. The resulting colonies consisted of yeast-like cells, which contained only one nucleus in contrast to the cells frequently obtained by intraspecific protoplast fusion of *Candida* species. Both the DNA content of the fusion products and the analysis of haploids induced by chemical and/or by UV-light suggested that they were aneuploids.

INDUCED INTERSPECIFIC PROTOPLAST FUSION IN FISSION YEASTS

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Protoplasts of the "four-spored" *Schizosaccharomyces pombe* and the "eight-spored" *Schizosaccharomyces octosporus* were fused. The protoplasts were obtained from the cells by cell wall lytic enzymes produced by *Trichoderma viride*. When a mixture of the protoplasts of complementary auxotrophic mutants was treated with 30% polyethylene glycol 4000, cell fusion and complementation was attained with a frequency of 10^{-5}–10^{-7}. The "interspecific" fusion products showing a transitional morphology existed only on minimal medium and, in the absence of nutritional pressure when complementation was no longer necessary, they segregated immediately into auxotrophic cells with the markers of *S. octosporus*. No segregants were found displaying the morphology or markers of *S. pombe*. This asymmetric segregation indicates that *S. octosporus* might be dominant in the fused cells and they probably did not accept the whole genome of *S. pombe* but only the nucleus or less, or even only one chromosome.

TRANSFER OF MITOCHONDRIA VIA ANUCLEATE PROTOPLASTS IN SACCHAROMYCES CEREVISIAE

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During protoplast formation in budding yeasts, protoplasts 1 μm or less in diameter are frequently released from the buds. Although the majority of these small protoplasts do not possess nuclei, they do contain mitochondria. Anucleate small protoplasts are easily separated from those containing nuclei by low-speed centrifugation. After fractionation less than 5% of

the small protoplasts contained nuclei. An anucleate protoplast fraction was prepared from an erythromycin resistant (ery^R) auxotrophic mutant of *Saccharomyces cerevisiae*. Its erythromycin resistance is determined by a mitochondrial gene. Polyethylene glycol and Ca^{++} were used to induce fusion between this small protoplast fraction and the nucleus-containing protoplasts of a neutral petite (rho^0) mutant derived from an adenine-requiring strain of *S. cerevisiae*. Under partially selective conditions, the majority of fusion products were haploid and ery^R, containing the nucleus of the recipient adenine-requiring strain and the mitochondrial genome from the ery^R respiratory-competent donor cells.

IMMUNOPEROXIDASE STAINING OF YEAST CELLS AND PROTOPLASTS

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For immunocytochemical investigation of yeast cells and protoplasts, an India-ink immune reaction and an immunofluorescence method have been developed. In the present work, utilization of an anti-rabbit IgG sheep-serum conjugated with horse-radish peroxidase is presented. Treating *Candida albicans* and *Saccharomyces cerevisiae* cells and protoplasts with an appropriate immune serum, with the above conjugate and with a buffered H_2O_2–diamino benzidine substrate mixture, a well-evaluable and highly sensitive staining can be achieved, which is improved with a Victoria blue contrast stain. High sensitivity, easy and cheap realization are the advantages of the method over indirect immunofluorescence. Utilization of the method is demonstrated in detection of *S. cerevisiae* cells and protoplasts, as well as in the differentiation of *S. cerevisiae* and *C. albicans* protoplasts, and also in the detection of *C. albicans* antibodies in patient sera.

INTERGENERIC PHYSICAL FUSION OF YEAST PROTOPLASTS

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Candida albicans and *Saccharomyces cerevisiae* protoplasts were induced to fuse and in the fusion products the first partner was detected with the authors' India-ink immuno reaction, utilizing anti-*Salmonella* O7 serum, which reacts specifically only with *C. albicans*. The *S. cerevisiae* part of the fusion product was visualized with safranine T staining. During subsequent incubation of the fusion product in a stabilizer, the *C. albicans* membrane antigens were seen to swim into the *S. cerevisiae* membrane, indicating membrane fluidity. Thus the method allows to observe the efficiency of the physical part of fusion, even in the case of genetic–metabolic incompatibility when neither regeneration nor colony formation occur. When *S. cerevisiae* protoplasts were prestained with rose bengal, during the incubation subsequent to fusion the dye was transferred into the *C. albicans* part due to cytoplasmic intermixing (the *C. albicans* part was again visualized with the India-ink immuno reaction). Additionally, when particles were introduced prior to fusion into *C. albicans* protoplasts, as *in situ* reduced tetrazolium violet, the marker was transferred into the *S. cerevisiae* part due to cytoplasmic intermixing.

PROTOPLAST FUSION BETWEEN POLYENE-RESISTANT AND SENSITIVE MUTANTS OF CANDIDA ALBICANS

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In order to study the mechanism of resistance to polyene antibiotics, nystatin resistant mutants were produced by nitrosoguanidine treatment from auxotrophic mutants of *Candida albicans*. Nystatin resistant and sensitive strains were characterized by cross-resistance to amphotericin B, candicidin, pimaricin, and cogomycin; ergosterol content; thin-layer chromatographic sterol spectrum; polyene-induced ion leakage; nystatin-induced ultrastructural alterations of the plasma membrane by freeze-etching electron microscopy; and staining of the cells by UO_2^{++} Ponceau-red after polyene treatment. The results indicate that resistance arises due to changes, either qualitative or quantitative, in the (ergo)sterol content of resistant strains. Polyethylene glycol-induced protoplast fusion was carried out between a sensitive (his^-, thr^-, met^-) and a nystatin-resistant (ade^-) strain, and heterokaryotic fusion products were obtained up to a frequency of 4.6×10^{-4}. Prototrophic, uninucleate, stable, ergosterol-containing hybrids which proved to be sensitive to nystatin, were isolated. The hybrid of the nystatin sensitive phenotype resulted in complementation to ergosterol production. Thus, the resistance to polyenes can be considered as a recessive character.

PENICILLIN PRODUCTION OF PENICILLIUM CHRYSOGENUM STRAINS OBTAINED BY PROTOPLAST FUSION

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Experiments were carried out with various auxotrophic and colour mutants of two *Penicillium chrysogenum* strains different in origin and fermentation capacity. PEG-induced protoplast fusion products were formed up to a frequency of 3.2%. From heterokaryons stable prototrophic progenies of larger conidial volume and of higher penicillin yield were isolated compared to the auxotrophic partners. One of them proved to be diploid. From the six other strains examined only one of the auxotrophic partners could be reisolated which suggests their aneuploid nature.

THE POSITION OF YEASTS IN THE SYSTEM OF FUNGI

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Significant changes in the systematization of living world have been induced by recent chemotaxonomic and functional cytologic results. Part of these concerned the position and sphere of the group fungi. In consequence of this (i) the group has obtained the rank of a regnum

equivalent to the *Plantae*, the *Animalia* etc., and (ii) its purging has been started, in addition to *Mycetozoa*, the taxa *Oomycetes*, *Plasmodiophoromycetes* and *Hyphochytridiomycetes* have been transferred to the regnum *Protista*. Within the regnum *Fungi* some additional rearrengements concerned the position of yeasts and yeast-like fungi. The position of yeasts could only be established in accordance with a rearrangement of the system of the whole regnum, and thus a proposal is made for partitioning the regnum *Fungi* into 7 + 1 divisions *(Chytridiomycota, Zygomycota, Zymomycota, Ascomycota, Taphrinomycota, Ustomycota, Basidiomycota* and *Deuteromycota)*. The heterogeneous group of yeasts and yeast-like fungi should be distributed among divisions 3, 5, 6, 7 and 8.

MELTING FINE STRUCTURE OF NUCLEAR DNA OF FILAMENTOUS FUNGI

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Numerical analysis of the thermal denaturation profiles of DNA is a useful tool for examining local fine-structural features or melting modes. The melting modes reflect the denaturation of a segment (or thermosome) inside the DNA molecule. Thermal denaturation of *Fusarium culmorum* nuclear DNA was performed by automatic and continuous increase of the temperature by 0.25 °C/min; denaturation at 260 and 270 min was recorded at full equilibrium (45–95 °C). Under such conditions, the melting curve (monitoring parameter vs temperature) exhibits reproducibly fine structural features or melting modes. A better visualization of the melting modes was achieved by recording the melting data (temperature, optical density) automatically and transformation into derivative form by a numerical differentiation program with a Hewlett–Packard HP-97 S Programmable Printing Calculator. According to numerical data, the differential form of the thermal denaturation profile of nuclear DNAs was represented by a graphical method. In this way at least 40 significant peaks (or thermosomes) can be identified in the thermal denaturation profile of fungi nuclear DNA. Values for the parameters (T_m, $\%G + C$, ΔT, S) of the most significant peaks were determined.

ULTRASTRUCTURE OF THE MACROCONIDIUM OF MICROSPORUM GYPSEUM

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The wall of macroconidia consists of four layers, i.e. from outside to inside, a strongly electron-scattering surface layer covered by significant warts; external granular layer; filamentous layer; and an internal granular layer. At the origin of the septa, the internal granular layer is homogeneous, while the filamentous one dividing into two parts bends inside and composes the septum. A tripartite space bordered by a triangle, is composed by the bifurcate bending-in of the filamentous layer from two opposite sites. On the septa, pores could not be seen directly but the inwards tapering of septa and the presence of Woronin-bodies indirectly indicate their presence. In the area of the triangle and in the other parts of the wall electron-scattering, irregular inclusion-like bodies, not delimited by a membrane, are to be seen, which have not been detected in other fungi. The plasma membrane is two-layered, similarly as that of the

hyphae and corresponds to the unit membrane. The presence of numerous small lipid-bodies among the cytoplasmic organelles bordered by a simple membrane seems characteristic. Microbodies were infrequently observed.

KARYOLOGY OF DERMATOPHYTES

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The vegetative hyphae and vegetative reproductive (macro- and microconidia) of dermatophytes were investigated using many kinds of nuclear-staining methods, different fixatives and staining solution. The intersepta of the vegetative hyphae of *Epidermophyton floccosum*, *Microsporum gypseum*, *Trichophyton ajelloi*, *Trichophyton vanbreuseghemii* and *Trichophyton quinckeanum* contain 1–2 (rarely 8–12) nuclei. In their microconidia 1–2 nuclei can be seen. In the intersepta of macroconidia of *E. floccosum*, 1–4, of *M. gypseum* numerous, of *T. ajelloi* 1–5, of *T. quinckeanum* 3–5 and of *T. vanbreuseghemii* 3–8 nuclei can be detected.

THE MORPHOGENETIC EFFECT OF CYSTEINE ON DERMATOPHYTES

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Twenty-four strains belonging to 21 dermatophyton species were investigated on culture medium for morphogenetic changes inducible with cysteine. None of the strains grew at 37 °C on a synthetic medium containing 0.06 M or more of cyteine. Important differences in tolerance to 0.01–0.02 M cysteine were detected among strains and species. The cysteine concentration allowed the strains to grow; growth was slow and the morphology was mostly shifted towards a glabrous, waxy surface. The morphogenetic effect of cysteine manifests itself microscopically with induction of the production of chlamydospore-like features on the culture medium surface region of the colonies. The cell-wall of the "chlamydospores" is thick and with and without Congo red staining it is anisotropic in polarized light. The chains of "chlamydospores" may resemble bud-chains and pseudohyphae, but real budding forms were not seen. Injecting intraperitoneally to mice of suspensions grown on cysteine-containing medium, solitary budding cells and/or chains of them were seen in PAS-stained smears of the peritoneal exudate taken from animals sacrificed on the 10–15th day.

2-DEOXY-D-GLUCOSE INDUCED MORPHOLOGICAL AND CHEMICAL CHANGES OF THE CELL WALL IN RHODOSPORIDIUM TORULOIDES

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2-Deoxy-D-glucose (DG) in the growth medium caused marked morphological changes of the cells of *Rhodosporidium toruloides*. The originally elongated ellipsoidal cells of the α strains grew spherically, showed incomplete cell division and separation, and were larger than the

control cells. Ultrathin sections revealed that the wall of cells grown in the presence of DG was thinner with somewhat less distinct contours than the wall of the control cells. The effective glucose to DG ratio (4 : 1–1 : 4) was much lower than for *Saccharomyces* and *Schizosaccharomyces*. After exhaustion of the glucose in the medium the cells died, although practically no lysis was observed. The morphological changes were probably a consequence of the alterations in the carbohydrate composition of the cell wall: it contained higher proportions of chitin and glucan, while the relative contents of mannan and galactose polymers decreased drastically.

STAINING OF YEASTS — VITAL AND POSTVITAL

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Forty-four histological and indicator dyes were examined in 0.6 M KCl for vital staining and for staining after formaldehyde or cetylpyridinium bromide treatment of *Candida albicans* and *Saccharomyces cerevisiae* cells and protoplasts. Some dyes of the anionic type stained neither the native cells not the treated ones. The native cells took only certain dyes, and in some cases qualitative differences were observed between the two species. The dyes staining the treated but not the native cells allowed in some cases a differentiation between stained protoplast and unstained native cells and/or protein denaturation (formaldehyde treatment) or membrane damage (cetylpyridinium bromide treatment).

A MODEL OF THE CELL

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For the interpretation of the results of cell multiplication and its inhibition a basic mathematical model of the elementary living system (living being + its relevant environment) has been constructed. In the model, the living system is considered a closed system, while the "living being" inside it as an open one. As to the behaviour of the "living being", the constant intrinsic density, i.e. the constant concentration of the living material in the open part-system is also postulated. The substrate of environment is partly decomposed by the "living being" (yielding energy), and is partly incorporated into the living material (from which it can again be composed). The stationary life of the "living being" is substrate-dependent (maintenance), but with a substrate supply above the maintenance limit it attains an exponential growth (balanced growth). The growth rate follows with a lag the increase or decrease of substrate concentration. At a substrate supply below the maintenance limit, the "living being" decomposes and dies. The postulated constant surface-volume ratio forecasts the change in form, or a division of the " iving being". Stimulations made with suitable parameters result in curves of microbial growth. Although the model is based on simple chemical reactions and does not include substrate pools and enzymes, nevertheless it gives good approach to cell (microbe) multiplication and implies also the models of von Bertalanffy, Perret and Monod as marginal cases.

ANTIFUNGAL PRESERVATION OF PHARMACEUTICAL PRODUCTS

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The efficiency of preservatives was studied on fungal suspensions and pharmaceutical products. Nipagin and sorbic acid were weakly fungicidal against *Candida albicans* and *Saccharomyces cerevisiae*. Nipasol killed at a somewhat higher rate, only the latter species. For the inefficiency neither the decomposition nor the detoxication of the preservatives is responsible. For the protection of insulin artificially contaminated with *C. albicans* and *Aspergillus niger*, the combination of Nipagin + Nipasol proved ineffective, even bacteria introduced artificially showed a transitory growth. A combination of veratric acid methyl ester + high-dose Nipagin gave better results. The natural or artificial fungal contaminations of prednisolone ointment could hardly be suppressed or eliminated, thus a decrease in prednisolone content was frequently observed.

TRANSMISSION OF ZEARALENONE AND ZEARALENOL INTO PORCINE MILK

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Sows were kept on a diet containing 40 mg/kg crystalline zearalenone from the 8th day after parturition, for 9 days. The vulva of their suckling female piglets became swollen and red on the 4–5th day of the experiment, then gradually the swelling progressed. Laboratory analysis showed the presence in the milk of zearalenone, though in an altered form. Beta-zearalenol was found in the largest quantities (82–86%), whereas alpha-zearalenol and zearalenone occurred in smaller quantities (13–17% and 0.5–1.3%, respectively). The highest concentration of zearalenol found in the milk was 0.51–0.79 mg/kg. The toxin was detected in the milk samples as early as 42–44 hr after starting the diet, and its residue persisted for 5 days after discontinuing the diet.

PRODUCTION OF PORPHYRIN BY CANDIDA GUILLIERMONDII VAR. GUILLIERMONDII STRAINS

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Porphyrin synthesis by animal-pathogenic and standard *Candida guilliermondii* var. *guilliermondii* strains was studied. The strains were found to synthesize, besides the precursors, all members of the 2–8 carboxylic porphyrins, the uro III and copro III isomers being the dominating ones. The presence of serum in the culture medium is favourable for multiplication of the strains, but porphyrin production is less intensive in serum-containing medium, presumably due to the feed-back inhibition by haeme.

SPOROTRICHOSIS OF RARE LOCALIZATION

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Inflammation of the lachrymal gland accompanied by conjunctivitis caused by the dimorphic fungus *Sporothrix schenckii* is reported. The infection was probably acquired occupationally and provoked by cortisone treatment. The following points merit attention. Sporotrichosis is a rare disease in Hungary; sporotrichotic infection of eyes is infrequent conjunctival localization has not been observed in Middle Europe; involution of the lachrymal gland has never been reported.

Agricultural and Industrial Microbiology

EFFECTS OF PESTICIDES ON NITROGEN FIXATION (C_2H_2 REDUCTION) BY AZOTOBACTER CHROOCOCCUM AND AZOSPIRILLUM LIPOFERUM

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The effect of pesticides was studied on nitrogen fixation by aerobic soil bacteria growing in semisolid media with 0.2% agar where the oxygen supply was limited by diffusion. *Azotobacter chroococcum* was grown on a N-free salt glucose medium, *Azospirillum lipoferum* in Döbereiner's malate medium at 30 °C for 1 week with and without addition of commercial pesticides. The herbicides dinosebacetate (Aretit, Hoechst) and ioxynil (Trevespan, Cela) were added at concentrations of 20 and 100 $\mu g \cdot ml^{-1}$ of active ingredients, the herbicide paraquat (Grammoxone, Cela–Merck) and the fungicide dodine (Questuran, Urania) at concentrations of 20 $\mu g \cdot ml^{-1}$. Acetylene reduction was measured for 60 min in 12 replicate tubes after 2, 4 and 7 days of incubation. Depending on pesticide and organism, four different responses were observed. (i) No inhibition and/or stimulation (*A. lipoferum* with dinoseb and ioxynil); (ii) initial inhibition was reverted into strong stimulation (*A. chroococcum* with ioxynil and dodine, *A. lipoferum* with dodine); (iii) initial stimulation reverted to strong inhibition (*A. lipoferum* with dodine) and (iv) strong inhibition persisted throughout growth (*A. chroococcum* with paraquat). Activities of cultures were not modified by repeated acetylene reduction tests.

ADSORPTION OF NONIONIC TENSIDES ON THE SURFACE OF CORONILLA–RHIZOBIUM K40 AND K59 STRAINS

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Nonyl-phenyl-ethylene oxide polymers belonging to the nonionic surfactants show a marked inhibitory effect on microorganisms or enhance the biological activity of other xenobiotics. To study the correlation between microbicidal effect and chemical structure, the ad-

sorption of the nonyl-phenyl-ethylene oxide containing 4, 5, 6, 8, 9, 10, 13, 15, 23 and 30 ethylene oxide groups per molecule was measured after 5, 15, 30, 45, 60 and 90 min incubation at 150 ppm concentration. The adsorption after 5 min of nonionic tensides on the surface of *Coronilla–Rhizobium* cells in μg/ml in the above order of ethylene oxide groups per molecule was for strain K40, 132.7, 107.2, 109.8, 103.5, 99.5, 101.5, 100.5, 103.2, 101.2, 101.9, 103.8, for strain K59 129.9, 100.6, 102.7, 108.4, 103.8, 96.1, 104.6, 95.1, 64.0, 79.7, 97.7. After 5 min the adsorbed amount showed no significant change, i.e. the time of surface saturation was less than 5 min.

COMPARATIVE INVESTIGATION OF CORONILLA–RHIZOBIUM STRAINS

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Rhizobium strains were isolated from root nodules of wild types and cultivars of *Coronilla varia* L. occurring in different soil types and cultivated in different experimental treatments. Fifteen of the 31 pure cultures maintained, were selected for detailed examination. (i) *Coronilla-Rhizobium* strains belong to the "fast growing" rhizobia differentiated from taxonomical and inocula production points of view. (ii) On sandy soil 45 kgN/ha dose was favourable for the formation of *Coronilla-Rhizobium* symbiosis, but doses higher than 90 kg/ha decreased the nodule formation. (iii) Among the 8 fungicides studied Germisan and Falisan, containing Hg harmful to the environment, exerted the strongest inhibition on the examined 15 strains of *Rhizobium*. Strains of 15 *Fusarium* species, considered as potential pathogens for *Coronilla*, were more sensitive to fungicides than the rhizobia. The inhibitory effects for *Rhizobium* strains were fairly uniform, but differed significantly for different species of *Fusarium*.

THE ROLE OF SLIME-MATERIAL OF XANTHOMONAS VESICATORIA IN THE DEVELOPMENT OF BACTERIAL BLIGHT OF TOMATO

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Water soaking lesion is the first symptom which appears on leaves infected with plant pathogenic bacteria. In the case of some phytopathogenic pseudomonads, bacterial mucopolysaccharides are responsible for water soaking of the intercellular spaces of parenchymatous tissues. Production of slime material of a virulent and an avirulent strain of *Xanthomonas vesicatoria* pathogenic to tomato and pepper plants was studied together with the role of slime in the bacterial blight disease of tomato. It was found that virulent and avirulent strains both produced slime material in a similar quantity and the material injected into different host plants caused water soaking lesion on the leaves. These results suggest that the virulence of the pathogen is unrelated to the slime production of *X. vesicatoria* in comparison with other mucopolysaccharides isolated from phytopathogenic pseudomonads.

INFLUENCE OF NITROGEN FERTILIZATION ON PROTEOLYTIC ACTIVITY OF CERTAIN SOILS

V. Rankov, G. Dimitrov and V. Barov

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In model trials, under controlled conditions, the influence of fertilization with ammonium sulphate, sodium nitrate, ammonium nitrate and carbamide was investigated on the proteolytic activity of soils with different physico-chemical properties: leached chernozem, leached chernozem-smolnitza, leached meadow-cinnamonic and alluvial-meadow soil. Doses of 120, 240, 360, 480, 600 kg of N/ha were tested on the basis of $P_{240}K_{240}$ in the course of 90 days after fertilization. Changes in enzymic activity after fertilization with ammonium sulphate and ammonium nitrate were greater than those caused by sodium nitrate, and more expressed in alluvial-meadow soil than in leached chernozem and leached chernozem-smolnitza. The different nitrogen fertilizers had a similar influence on the development of ammonifying bacteria.

PATHOGENIC FEATURES OF STREPTOMYCETES

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The toxicity of two *Streptomyces* strains isolated from drinking water has been investigated for 240 days. A chloroform extract in oil of a culture of *Streptomyces venezuelae* was inoculated intramuscularly into white male rats weighing 119.5 $\pm$ 5.8 g. Doses of 1 mg/animal daily induced inflammatory myocardial lesions and hepatic and renal changes. The toxic substance obtained from *Streptomyces griseus*, administered under identical conditions, induced interstitial pneumonitis and testicular changes. It is supposed that in the *Streptomyces* genus there are several other species possessing toxic properties and it is necessary to clarify their significance in the context of human ecologic relations.

WILD YEASTS OCCURRING IN THE PROCESS OF BAKER'S YEAST PRODUCTION

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Different wild yeast strains infecting baker's yeast were isolated by the plating method. Investigations comparing the VDH strain of *Saccharomyces cerevisiae* with the infecting C I and C III isolated wild yeasts were carried out. Morphological studies showed that colonies of wild yeasts greatly differ from those of *Saccharomyces cerevisiae;* the cells of the former were smaller in size and formed pseudomycelia on potato-glucose agar. During fermentation in a 6 litre vessel, the generation time of the C III wild yeast was $t_g = 2.0$ hr, slightly less than that of VDH ($t_g = 2.3$ hr). Both VDH and C III showed effective proliferation on glucose and molasses. C I wild yeast rapidly proliferated on glucose ($t_g = 1.2$ hr) but it grew slowly on molasses having

no invertase activity. Mixed cultures of C I with VDH showed a better yield and a 1.5 times higher cell concentration than the pure culture of VDH. During cultivation the ratio of C I in the mixed culture increased from 5% to 57% and from 15% to 86%. C I was found to make use of the sucrose inverted by VDH. According to standard fermentation power, wild yeasts produce 50–70 ml CO_2 gas, whereas with average quality baker's yeast CO_2 development amounts to 700 ml in 60–70 min. Thus, isolated wild yeasts are unable to ferment sucrose and maltose.

INFLUENCE OF LOW TEMPERATURE STORAGE ON RAW MILK

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The influence on raw milk of low temperature storage has been studied. The bacteriological and chemical quality of raw milk was investigated during storage at 5, 7 and 10 °C for three days. The counts of mesophilic, psychrotropic thermophilic, thermoduric, proteolytic and coliform organisms were determined and the acidity, pH, and coagulation by alcohol were investigated. The counts of mesophilic and psychrotropic organisms increased at 5, 7 and 10 °C more than those of the proteolytic and coliform organisms. The thermoduric organisms increased the least while the counts of thermophilic bacteria did not change during the 3-day storage. Storage of raw milk at low temperatures caused chemical changes. The pH varied with temperature and storage time; at 5 °C, it decreased slightly during the first 2 days and strongly on the third day; at 7 °C the pH decreased after 1 or 2 days. At 10 °C, the decrease was more marked than at 5 °C and 7 °C. Alcohol test was negative at each temperature, only at 10 °C were some samples coagulated.

MICROBIOLOGICAL INVESTIGATIONS INTO SODIC LEACHING OF MECSEK URANIUM ORES

B. Czeglédi, K. Czakó—Vér, L. Fekete and M. Kecskés

Mecsek Ore Mining Company, Pécs and Research Institute of Soil Science and Agricultural Chemistry, Hungarian Academy of Sciences, Budapest

At the Mecsek Ore Mining Company prism percolational leaching technology has been used for processing ores with low uranium content. (i) In the sodic uranium leaching system, at pH 9.0–9.5 the number of heterotrophic bacteria was 10^3 cells/ml, and that of chemoautrotrophic bacteria 10^5–10^6 cells/ml. (ii) The number of chemoautotrophic bacteria was 10^6 cells/ml at 85 g/litre Na_2CO_3 concentration. (iii) Declining from the top of 8–12 m high percolational prisms the "total" number of bacteria decreased, then increased again in the lower drainage level. (iv) In the prisms having proper yields and good percolation ability, bacterial counts were considerably high (10^7/g) whereas under bad percolation conditions and at less advantageous yield, relatively low (10^3–10^4/g). (v) In order to identify bacteria, contributing to uranium yield actively, different isolates were collected from the mining water, surface water and from ore samples. The majority of isolated bacterial strains belonged to the *Thiobacillus* genus, having weakly alkaline or neutral pH optimum. In characteristics they differed considerably from representatives of *Thiobacillus ferrooxidans* applied in hydrometallurgy so far.

EFFECT OF SOME ABIOTIC FACTORS ON THE FE (II) OXIDATION ACTIVITY

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Geochemistry Research Laboratory, Hungarian Academy of Sciences, Budapest, Research Institute for Plant Protection, Budapest and Research Institute for Soil Science and Agricultural Chemistry, Hungarian Academy of Sciences, Budapest

Fifteen strains of *Thiobacillus ferrooxidans* were selected for eco-physiological investigations from strains isolated from copper, iron, manganese and coal mines as well as surface waters (stream and spring). These strains were compared with four authentic strains of *T. ferrooxidans* in stationary liquid cultures. The strains were compared to each other according to Fe(III) production, i.e. their oxidation capacity. Significant differences were found among the strains isolated at the same site, too. Studying the dependence of Fe(II) oxidation activity and the seed of redox potential change on the temperature in 4–45 °C interval, the optimum was between 20–28 °C. Four strains were recorded with values higher than this, namely 37 °C. The oxidation catalyzed by bacteria could not be measured at 4 and 45 °C. As regards the pH sensitivity of strains on the basis of Fe(II) oxidation speed in the pH 1.0–3.0 interval, the maximum pH value was pH 2 pH 2.5 for 2 strains. Similarly the strains could be grouped on the basis of pH dependence of redox potential of fermentation liquids. The pH and Cu(II) concentration had a combined effect on the Fe(II) oxidation speed of the strains. The Cu(II) tolerance could be characterized as a hyperbolic function varying markedly with the strains. To prove the applicability of the isolated strains for leaching of ores, the percolation test and "fast" leaching of calcoperit of Recsk were carried out.

MICROBIOLOGICAL DEGRADATION OF OIL POLLUTION OF SOILS

F. Buday, L. Sass, G. Török, Z. Gergely, Á. Szakács and I. Vörös

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Microbiological degradation of oil pollution has been studied with mixed cultures, isolated from oil polluted areas, in which *Nocardia*, *Pseudomonas*, *Flavobacterium* and *Candida* species dominated and which decomposed 80% of the oil added to the culture medium under laboratory conditions in 5 days. Large scale experiments were set up using different types of oils and oil sludges. In the inoculated areas, 70–90% of the oil, depending on its quality, was rapidly degraded in the soil. For determining the residual part, gas chromatography and infrared spectrophotometry were applied. It was observed that the n-paraffins practically disappeared from the residue. Infrared spectroscopy revealed in the residue many oxygen-containing compounds, and this fact confirms its advanced oxidation.

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AQUATIC BACTERIA AND FUNGI IN DANUBE RIVER AND IN THE WATER PRODUCING SYSTEMS OF THE BUDAPEST WATERWORKS

T. HORTOBÁGYI

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(Received March 29, 1979)

Of 635 microorganisms belonging to different taxa isolated in the years 1968–1976 from Danube water in the Budapest region and from water-producing installations, 13 bacteria and 1 fungus (*Schizomycophyta* and *Mycophyta*) are described. A new genus (*Caulastrum* gen. nov.), two new species (*Caulastrum danubiale* Hortobágyi sp. nov., *Planctomyces hajdui* Hortobágyi sp. nov.) and a subspecies (*Planctomyces crassus* Hortobágyi subsp. *maximus* Hortobágyi subsp. nov.) are defined. Planctomycetes belonging to the alpha-mesosaprobionts were represented by a great number of taxa and occurred in gradually increasing numbers associated with the increasing pollution of the river. Of these organisms *Planctomyces békefii* Gimesi was the most prevalent, *Planctomyces crassus* Hortobágyi and *Planctomyces gracilis* Hortobágyi were next in order. Other species occurred in greater numbers only in certain samples.

In the years 1968 to 1976, I examined the microflora of the Budapest reach of the Danube (river kilometers 1586–1654) and of the waterworks utilizing Danube water.

The present paper deals with the microorganisms belonging to the phyla *Schizomycophyta* and *Mycophyta* from the isolated 635 taxa. Of the 14 organisms descibed 13 are bacteria, 1 fungus.

Treated are 1 genus: *Caulastrum* gen. nov., 2 species: *Caulastrum danubiale* Hortobágyi sp. nov., *Planctomyces hajdui* Hortobágyi sp. nov. and 1 subspecies: *Planctomyces crassus* Hortobágyi subsp. *maximus* Hortobágyi sups. nov.

The high number of taxa of 7 *Planctomyces* belonging to the alpha-mesosaprobionts and their increasing proliferation during the investigation period was remarkable; it indicates the river's increasing pollution.

The taxonomy applied does not entirely correspond to that used in Bergey's Manual of Determinative Bacteriology, 8th ed., 1975. The water samples at issue are at the Botanical and Phytophysiological Department of the University of Agricultural Sciences at Gödöllő.

Materials and methods

In the years 1968 to 1976, on the request of the Budapest Waterworks, a team (leader Dr. Á. BERCZIK) examined the Danube water between the river kilometres 1586 and 1654, the reach being the feed-water of the water supplying installations, further the horizontal,

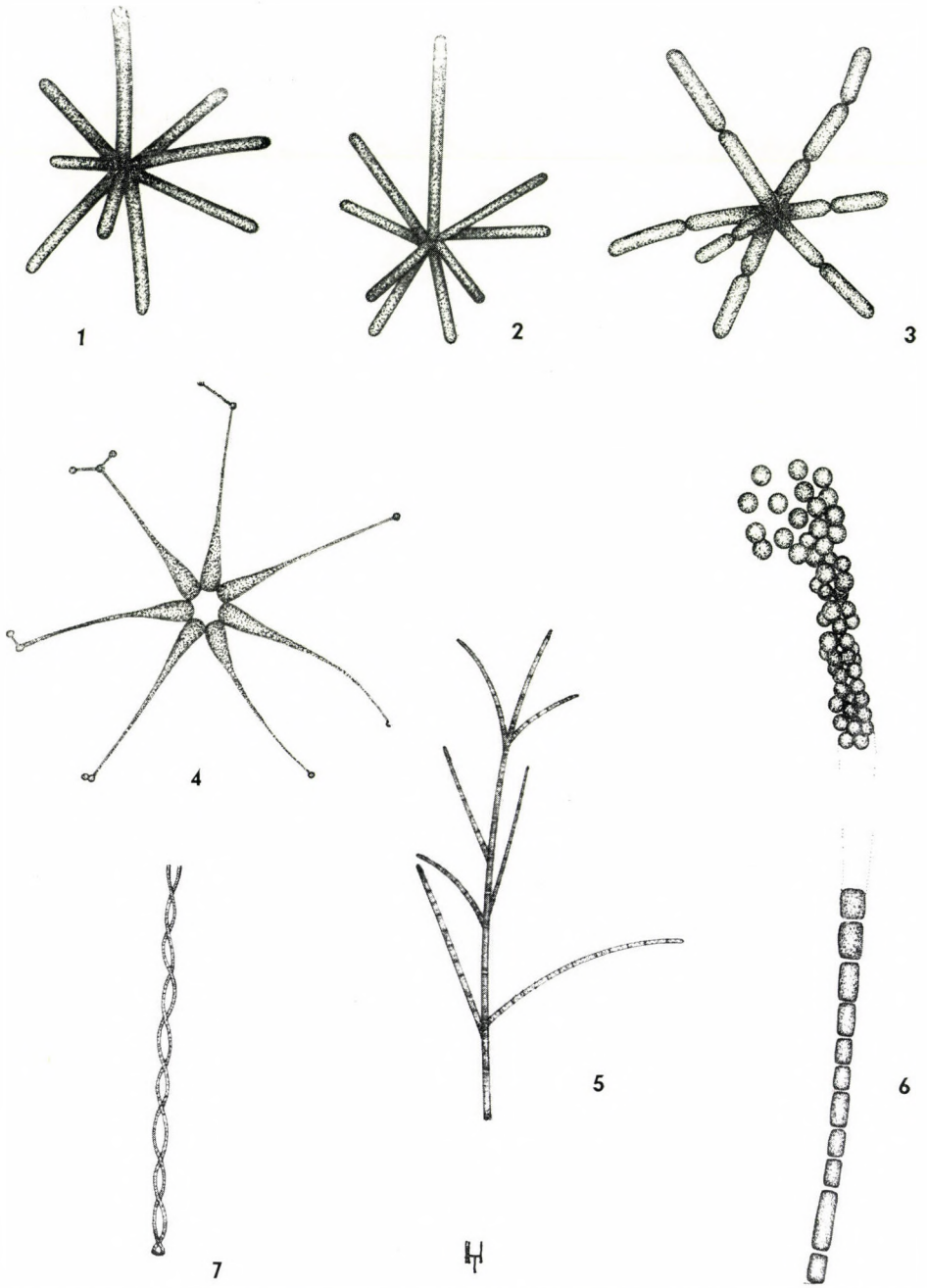

Plate I. Figs 1—3. Agrobacterium stellulatum Stapp et Knösel; *Fig. 4. Caulastrum danubiale* Hortobágyi; *Fig. 5. Crenothrix fusca* Dorff; *Fig. 6. Crenothrix polyspora* Cohn; *Fig. 7. Gallionella ferruginea* Ehrenberg

test, dug or productive wells, the test drillings, the settling and enriching basins and the reservoirs, from the aspects of general hydrobiology, ecology, taxonomy and saprobiology. My task was to examine qualitatively and partly quantitatively the microflora of the dipped water samples. The Enumeration section of this paper lists only the sampling sites where the organisms described could be detected. The dipped water samples were preserved in 2% formalin and examined by phase contrast microscopy with immersion objective.

During the 9-year period, 635 taxa of the phyla *Schizomycophyta, Mycophyta, Cyanophyta, Euglenophyta, Chrysophyta, Pyrrophyta* and *Chlorophyta* were determined. The present paper discusses the 13 taxa belonging to the phyla *Schizomycophyta* and *Mycophyta;* among them, there were 12 bacteria and 1 fungus.

Ecological notes

The high number of taxa (7 of *Planctomyces)* that can be regarded as alpha-mesosaprobionts and their continuously increasing proliferation in the 9-year period was remarkable. It was especially the year 1975 when their number per litre was remarkably high, but they were frequent both in the previous years and in 1976. The most frequent were the morphotypes with slender and thicker stalks of the species *Planctomyces békefii* Gimesi demonstrable in the Danube, the Soroksár Arm of the Danube, the settling and enriching basins and in the test wells. The organisms proliferated mostly in the Danube, the Soroksár Arm of the Danube and in the basins. The next frequent organism of the *Caulobacteriales* was *Planctomyces crassus* Hortobágyi. It was more rare in the Danube, though at times the number was unexpectedly high as e.g. in August, 1971, and September, 1975. The organism occurred frequently in the Soroksár Arm of the Danube in August, 1972.

Among the *Planctomyces, Planctomyces gracilis* Hortobágyi was the third in order of frequency. On one occasion, in June, 1975, a high number of coenobia was detected in the Danube. Many individuals of *Planctomyces guttaeformis* Hortobágyi (Danube, July, 1973) and of *Planctomyces hajdui* Hortobágyi (Danube, September, 1975) occurred in one collection each. The considerable proliferation of *Planctomyces* indicates the increasing pollution of the river.

Enumeration

Schizomycophyta

1. *Agrobacterium stellatum* Stapp et Knösel 1954 (Plate I, Figs 1—3).

In my book published in 1973, I described the species under the name "*Hyphomycetes* sp. I" (p. 53, Fig. 10). More recent observations allowed its determination and a clarification of its dimensions.

The cells are colourless, straight, rod-shaped, the ends are rounded, sometimes somewhat curved. The cells are 3–12 μm in length, approximately 1.0 μm in breadth, they radiate from a common base to form rosettes. The

colonies are radial. I have observed their reproduction by dichotomy; the cells are constricted perpendicularly to the longitudinal axis.

The specimens were obtained from the Danube, Danube river kilometre (henceforth rkm) 1674 from November, 1968 to October, 1971 and the enriching basin of the Budapest Waterworks in October, 1971.

Caulastrum gen. nov. Hortobágyi (caulis = stalk, aster = star)

The cells, broadly rounded at their base, are attached to each other on a small surface. They end gradually attenuating in blunt tips. There is a cavity formation at the attachment. The cells are arranged in plane. Reproduction occurs by the conidia separating from the end of the cells. Type:

2. *Caulastrum danubiale* Hortobágyi sp. nov.; Syn. *Planctomyces actinastroides* Hortobágyi; danubiale = from the Danube (Danubius) (Plate I, Fig. 4)

The much elongated drop-shaped cells have a broadly rounded base, the tips are blunt. They are straight or somewhat curved, the contact at the base is restricted to a very small surface, where a cavity is formed. The colourless cells are arranged in a plane. They measure 11.7–14.3 μm in length, and at the base 1.5–2.0 μm in breadth. The spores develop at the ends facing outward, they are spherical in shape, 0.3–0.5 μm in diameter. They separate from the mother plant but more frequently there is a budding process on the mother plant from where the spores are released. When describing the species in 1973 under the name "*Planctomyces actinastroides* Hortobágyi" (p. 52, Fig. 11), I already mentioned that it differed from the typical *Planctomyces*, since the cells did not form a radial cluster but were arranged in one plane. A further difference is the large cavity at the attachment of the cells, which cannot be observed in typical *Planctomyces*. The planar arrangement of the cells and the cavity formation justify a differentiation.

Specimens were obtained from the settling basin of the Budapest Waterworks fed with Danube water, in July and August, 1969.

3. *Crenothrix fusca* Dorff 1934 (Plate I, Fig. 5)

The branching filaments measure 5.0–7.0 μm at their base. The upper attenuating ends are 1.5 μm in breadth. The cells are 2.0–6.0 μm long. The sheath is colourless or brownish-red. They are alone or in pubescences.

Specimens were obtained from the horizontal well alongside the Danube in August, 1971.

4. *Crenothrix polyspora* Cohn 1870 (Plate I, Fig. 6)

The trichoma gradually broadens; at the base it is 2.5 μm in breadth, at its end it measures 5.0 μm. Usually no branching occurs. The cells in the filaments are mostly cylindrical and 3.0–7.5 μm long. The diameter of the spherical cells released from the trichoma measures 1.5–3.0 μm.

Specimens were derived from the horizontal well along the Danube in June and August, 1971; from the test well in October, 1971; from the test well and horizontal well in May, July and August, 1973 and from the reservoir in November, 1975.

5. *Gallionella ferruginea* Ehrenberg 1838 (Plate I, Fig. 7)

The trichoma is 0.3–0.5 μm in breadth. It is colourless or light yellow symmetrically twisted.

Specimens were derived from the horizontal well in December, 1971, and October, 1972; from the Soroksár Arm of the Danube in August, 1972; from the test and horizontal wells in May, July, August and October, 1973; from a test drilling in July, 1973; from the dug well or productive well in July and October, 1973; and from the reservoir in November, 1975.

6. *Planctomyces békefii* Gimesi 1924 (Plate II, Figs 8–11)

The species is varied in structure, the rich colonies grow at a high rate. The stalks are slender, straight or slightly curved. The spherical cells found at the end of the stalks are 1.0–1.6 μm in diameter. It is the most frequent *Planctomyces* in the wells, the basins and the Danube.

Specimens were obtained from settling and enriching basins in August to October, 1968, from settling and enriching basins in June to September, 1969; from the Danube 1674 rkm in July, 1970; from the Danube rkm 1674 in May, August and October, 1971; from the settling basin in August to September, 1971; from the Soroksár Arm of the Danube in August, from the settling basin in September, from the enriching basin in May and September, 1972; from the Danube rkm 1634 in July, from the test well in July, 1973; from the Danube rkms 1608, 1598 in May, rkms 1654 and 1633 in June, rkms 1628, 1623 1618, 1592, 1598, 1643, 1623, 1618, 1608, 1588 in September, 1975; from the Danube rkms 1654, 1638, 1630, 1628, 1623, 1613, 1603 in May, rkms 1654 to 1603 in August, 1976.

7. *Planctomyces békefii* Gimesi forma (Plate II, Figs 12–14)

The species differs from the prototype by its thicker stalks and generally smaller spores. The reproductive cells appear also here on the cells and either separate or bud while on the cells. The cells are 0.8–1.2 μm in diameter. The reproductive cells are sometimes perceivable while still included in the stalk.

In addition to the thicker stalks, the colonies have less stalks than does *Planctomyces békefii* Gimesi. In this species the number of stalks usually amounts to 5–6.

The coenobia are reminiscent of *Planctomyces crassus* Hortobágyi but here the stalks are much thicker and become broader towards the centre,

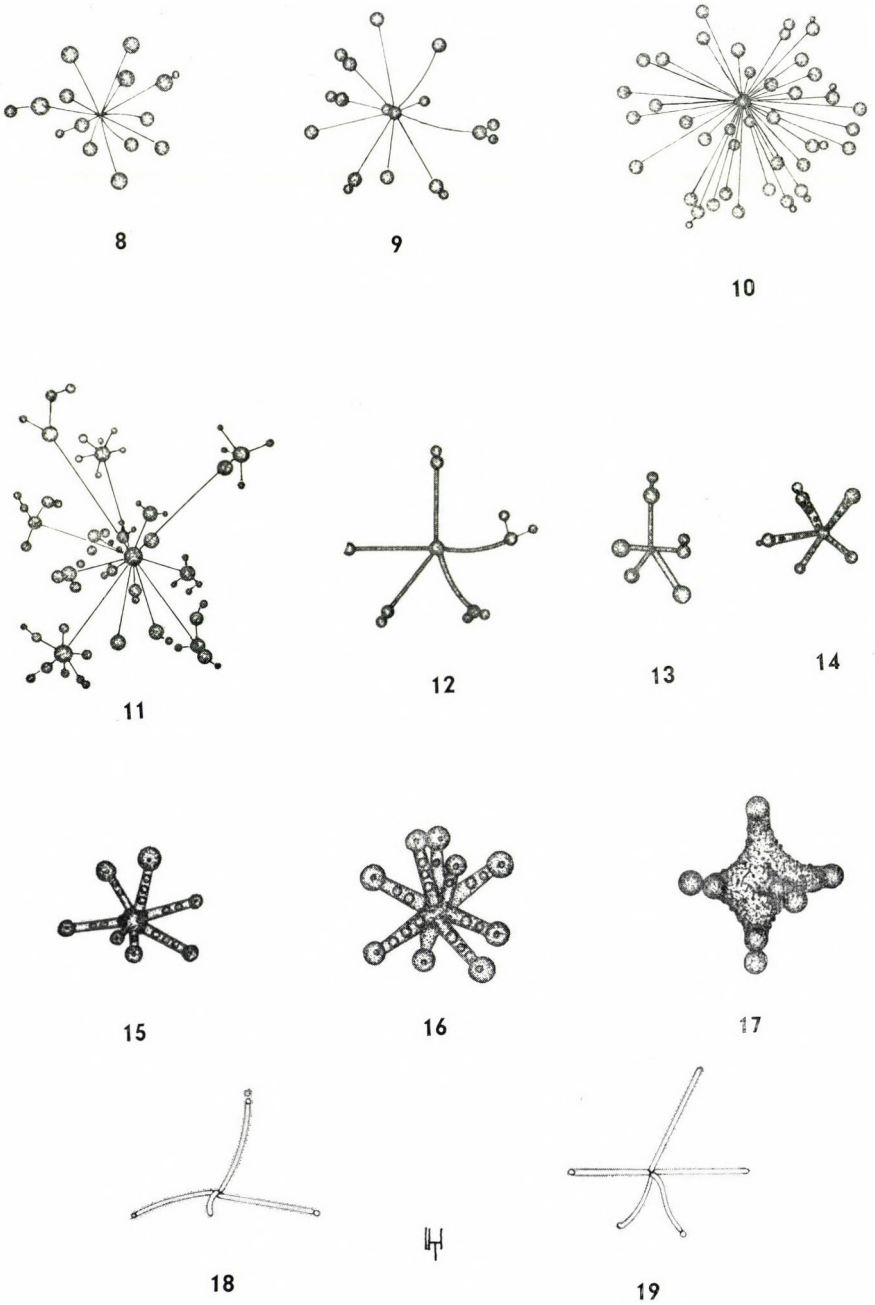

Plate II, Figs 8—11. Planctomyces békefii Gimesi; *Figs 12—14. Planctomyces békefii* Gimesi forma; *Figs 15, 16. Planctomyces crassus* Hortobágyi; *Fig. 17. Planctomyces crassus* Hortobágyi subsp. *maximus* Hortobágyi; *Figs 18, 19. Planctomyces gracilis* Hortobágyi

while in *Planctomyces békefii* Gimesi forma the stalks are slightly thicker only at the ends.

This type occurs side by with the colonies of the slender-stalked prototype and can be differentiated well.

Specimens were derived from settling and enriching basins in August to October, 1968, in June, 1969, and between 1970 and 1976 with the same frequency as the prototype.

8. *Planctomyces crassus* Hortobágyi 1965 (Plate II, Figs 15, 16)

The colonies are 9.0–13.0 μm in diameter. The stalks are thick and occur in greater numbers than in the coenobia of *Planctomyces békefii* Gimesi forma, they may number 10–12. The stalks become thicker towards the centre of the colony. The mature reproductive cells are 1.2–1.4 μm in diameter. Below the mature reproductive cells smaller ones are perceivable included in the stalks. Concretion may occur in the centre of the colony.

Specimens were derived from the settling basin in June, August and September, 1969; from the Danube rkm 1674 in August, 1971; from the Soroksár Arm of the Danube in August, 1972; from the Danube rkm 1623 in June, rkm 1633 in September, rkm 1598 in September, 1975; from the Danube rkms 1654, 1643, 1638, 1632, 1630, 1628, 1623, 1613, 1603 from May to August, 1976.

9. *Planctomyces crassus* Hortobágyi subsp. *maximus* Hortobágyi subsp. nov. (Plate II, Fig. 17)

The edges of the robust thallus have an uneven surface, they bend inwards, the side lengths are 5.2–6.0 μm. Thick somewhat tapering stalks, 1.5–2.0 μm long, grow from the thallus. Spherical cells and reproductive cells 0.8–1.2 μm in diameter grow at the end of the stalks. Younger reproductive cells are perceivable in the stalks.

The subspecies differs from *Planctomyces crassus* Hortobágyi in its robust thallus similar to that of *Planctomyces condensatus* Skuja, but its shape is different as is the development of reproductive cells.

Specimens were obtained from enriching basins in August, 1971.

10. *Planctomyces gracilis* Hortobágyi 1965 (Plate II, Figs 18, 19, Plate III, Figs 20, 21)

The stalks radiate from a common centre to from a rosette. Their length may attain 14 μm. They measure 0.3–0.4 μm in breadth and 0.3–0.4 μm in diameter, they are straight or arched.

Specimens were found in enriching basins in May, 1972; from horizontal and test wells in May, July, August and October, and from dug wells in April to October, 1973; from the Danube rkm 1598 in June, 1975.

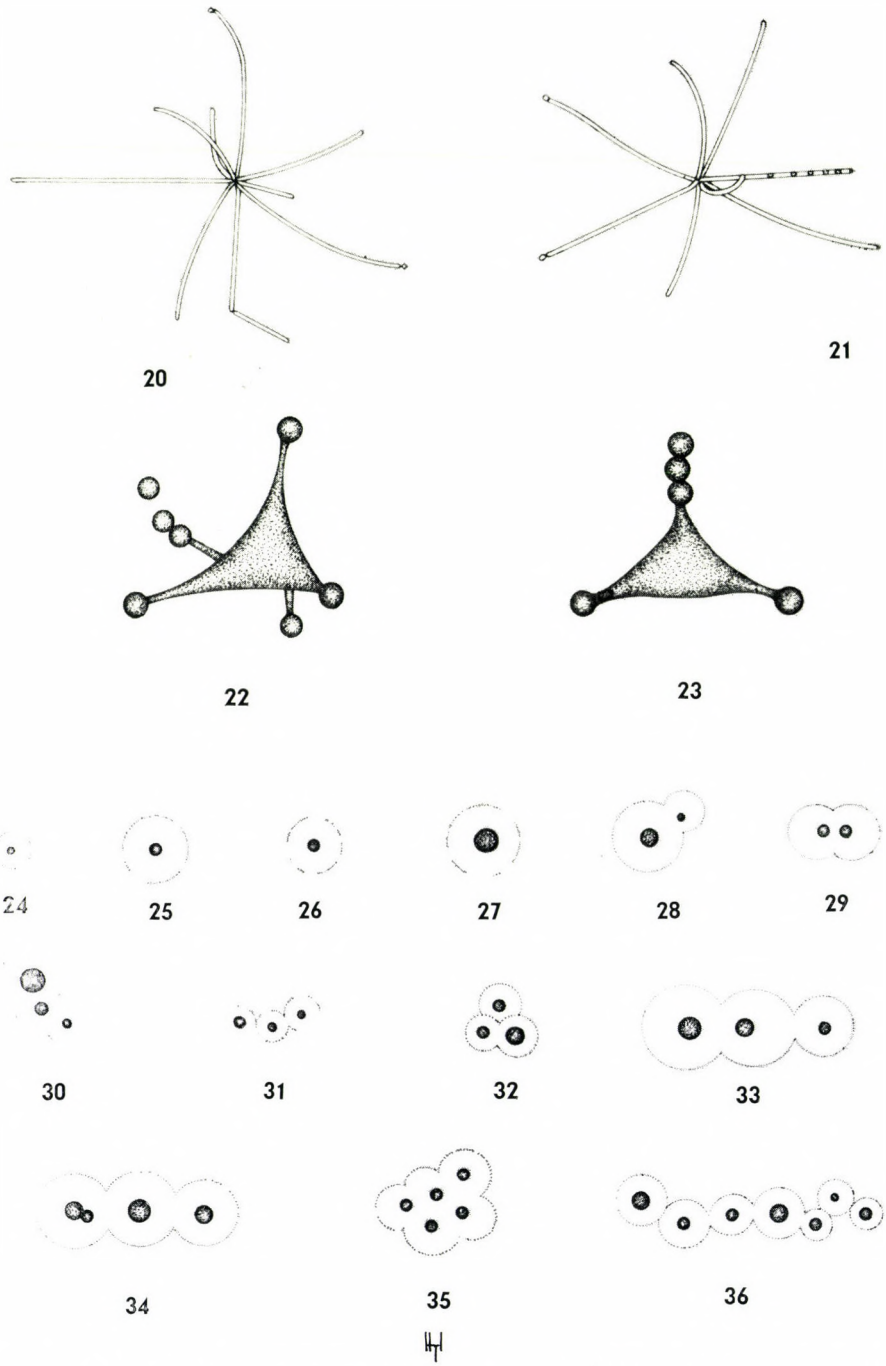

Plate III, Figs 20, 21. Planctomyces gracilis Hortobágyi; *Figs 22, 23. Planctomyces hajdui* Hortobágyi; *Figs 24—36. Siderocapsa coronata* Redinger

11. *Planctomyces guttaeformis* Hortobágyi 1965. Specimens derived from the Danube rkm 1634 in July, 1973.

12. *Planctomyces hajdui* Hortobágyi sp. nov. (Plate III, Figs 22, 23)

The colonies have 3–5 stalks. They are triangular in shape, the edges are concave or slightly convex, their surface is smooth, their length is 6.0–10.0 μm. The stalks branching from the thallus are tapering. Spherical reproductive cells develop in the stalks, the mature cells are 0.6–1.0 μm in diameter.

Specimens were obtained from the Danube rkm 1654 in August, 1974; and from the Danube rkm 1643 in September, 1975.

The species is nearest to *Planctomyces crassus* Hortobágyi but differs from it in its broad thallus and the projecting thinner stalks. Species dedicata in honorem doctoris Lajos Hajdu.

13. *Siderocapsa coronata* Redinger 1931 (Plate III, Figs 24–36)

The cells are spherical, 0.5–1.3 μm in diameter. They number 1–7 perceivable in a broad, colourless mucilage sheath, 2.0–8.0 μm in diameter.

Specimens were obtained from the Danube rkm 1674 in October, from enrichment basins in May and August, from horizontal wells in May and August from test wells in July, 1971.

Mycophyta

14. *Tetracladium marchalianum* Wildeman 1839 (= *Asterothrix rhaphidiodes* Printz)

Specimens derived from horizontal wells in June, 1971.

Diagnoses

Caulastrum gen. nov. Hortobágyi

Cellulae ad basis late rotundatae, versus apicem obtusum gradatim tenuiescentes, parte basali vix inter se contingentes, in uno plano dispositae; inter partes basales cellularum hiatus medius. Multiplicatio per constrictionem deinde abscissionem conidiorum in parte apicali cellularum.

Caulastrum danubiale Hortobágyi sp. nov. (Plate I, Fig. 4)

Cellulae hyalinae, guttuliformes, valde elongatae, basi late rotundatae hic parte valde parva inter se contingentes, hiatumque conspicuum in medio inter seipsos efformantes, apice obtusae, rectae vel parum inclinatae, 11.7–14.3 μm longae, ad basin 1.5–2.0 μm crassae, in uno plano dispositae. Sporae singulariter in apicibus cellularum extrorsis ortae, globosae 0.3–0.5 μm diam., aut — perumque — efformatae primo germinantes, dein discedentes, aut —

rarius — statim separantur. Jul.—Aug. 1969. In aquariis sedimentariis aqua fluminis Danuvii repletis. Officina Aquae Ductus Urbis Budapest (Fővárosi Vízművek, Budapest), Hungaria. — Raro.

Planctomyces crassus Hortobágyi subsp. *maximus* Hortobágyi subsp. nov. (Plate II, Fig. 17)

Thalli crassi, marginibus introrsis, lateribus 5.2–6.0 μm longis, superficie non levi, brachiis crassiusculis, in ipsis vel (et) in apicibus eorum sporis globosis, 0.8–1.2 μm diam.

Lacus septentrionalis accumulationis soli aquae causa constructus (Budapest), Aug. 1971. — Valde raro.

A *Planctomycete crasso* thallo crasso distinctus. Thallus subsp. novae nostrae illo *Planctomycetis condensati* Skuja crassitudine aequalis, sed forma eius et modo nascendi sporarum subsp. nova ab illo distincta.

Planctomyces hajdui Hortobágyi sp. nov. (Plate III, Figs 22, 23)

Thalli superne visu trigoni, superficie levi, lateribus concavis, in medio forte parum convexis, 6–10 μm longis, brachiis 3–5 versus apicem tenuiescentibus, sporas ferentibus. Sporae iam liberae 0.6–1.0 μm diam.

Danubius. Aug. 1974. — Raro.

Planctomyces crassus Hortobágyi ei proximus, sed ab eo thallo compacto, brachiis longis tenuibusque distinctus.

Species dedicata in honorem doctoris L. Hajdu.

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THE ROLE OF WILD BIRDS IN THE SPREAD OF INFLUENZA VIRUSES

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Eggs deposited by different migrating wild bird species in pond farm areas in Hungary were examined for yolk antibodies to different variants of human A/H3N2 influenza virus. Antibodies to Victoria/75 and Texas/77 occurred in 17.9 and 32.0% of gull eggs, and 5.6 and 16.4% of common tern eggs, respectively, while antibodies to A/H1N1/77 occurred in roughly similar proportions (10.2 and 13.4%) in the eggs of both species. Infection of the gull and tern populations of given areas by human and avian influenza A viruses differed greatly in two consecutive hatching periods. While in 1978 7.6 and 1.1% of the gull and tern eggs, respectively, contained antibodies to the avian subtype Havl, no such antibodies were found in 1977. Subtype A/H3N2/Texas/77 virus was isolated from adult gulls and 1–3 weeks old gull chicks, and subtype H1N1 virus from mallard ducks. Three months before the onset of the Texas/77 epidemic, 95% of SPF chickens, and 71–81% of chickens hatched 3 months after termination of the A/H1N1/77 epidemic, had had HI, VN and SRH antibodies to the Texas/77 strain and A/H1N1/77 strains, respectively.

Isolation of A/H3N2 variant Port Chalmers/73 from a wild bird — a collared dove (*Streptopelia decaocto*) captured in the premises of the Budapest Zoo — was reported for the first time by Romváry and Tanyi [1]. Immune response to the variant Victoria/3/75 was demonstrated in 29–40% of migrating wild birds as well as in 15.6 and 28.6% of zoo birds, representing 35 species. Among the collared doves, hooded crows (*Corvus corone cornix*) and house sparrows (*Passer domesticus*) shot and captured in farmlands along the river Tisza, 37.1 and 17.7%, respectively, proved to be positive serologically to Victoria/75. Isolation of the variant Victoria/75 from a sparrow captured along the river Tisza was also unprecedented in the literature [2]. Early in 1977, evidence was obtained of persistence of the variant Victoria/75 in a countryside Zoo, and in gulls and terns established for egg-laying on the peat marsh islets of a pond farm by presence of HI antibodies in sera and in the yolk of the eggs laid by the wild birds, and by isolation of Victoria/75 virus from a black-headed gull [3]. Serological tests and virus isolation showed that, in addition to Victoria/75, the gulls and terns had also become infected with the subtype Hav7 virus at a similar proportion. This virus was isolated from black-headed gulls and common tern, moreover a subtype Hav6 strain was isolated from a starling (*Sturnus vulgaris*) shot in a wild bird reservation area.

This prompted experimental studies of the nature of the influenza virus subtypes introduced by migrating wild birds into Hungary on their return from the Mediterranean area and Africa. In spring 1978, at the time of egg-laying and hatching, the eggs of common terns, black-headed gulls, and in a lesser number those of mallard ducks were collected along with one day and 1–3 weeks old gull chicks, moreover carcases of wild birds shot for examination to detect the possible presence of influenza virus(es).

Materials and methods

Virus isolation. This was attempted from a total of 93 wild birds resident in, or migrating through, a fish pond, a bird reservation area, and in different areas along the shore of river Tisza. Mainly the shot common terns, black-headed gulls and mallard ducks were taken into consideration as carriers of influenza viruses, but we examined for the presence of influenza viruses also ferruginous ducks (*Aythya nyroca*), pochards (*Aythya ferina*), moorehens (*Gallinula chloropus*), coots (*Fulica atra*), pheasants (*Phasianus colchicus*), hooded crows (*Corvus corone cornix*) and three sparrows (*Passer montanus*).

Samples were collected for virus isolation from the respiratory tract and cloacal mucosa. The homogenated samples were inoculated into embryonated hen's eggs pre-incubated for 9–11 days. The eggs were opened on the day of the embryo's death or 3–7 days after inoculation. Three blind passages were performed when required.

Virus strains. The A/H3N2/Victoria/3/75, the A/H3N2/Texas/1/77 reference strains, the A/H1N1/29/BP/78 strain, the avian influenza virus subtypes Havl–Hav9 reference strains, and antisera were used for identification of the isolates in the haemagglutination-inhibition (HI), single radial haemolysis (SRH) and virus neutralization (VN) tests.

Serum and yolk samples. The yolks of eggs laid by black-headed gulls, common terns and mallard ducks, and serum samples from wild birds shot for virus isolation, moreover sera of a few days to 1–3 weeks old gull chicks were also included in the serological screening.

Serological tests. The HI and VN tests were performed as recommended by the WHO in circular No. Z2/180/11/67. The SRH test, a new method for the assay of antibody to influenza haemagglutinin was done as described by SCHILD *et al.* [4]. Antibody in the egg yolks was detected as proposed by CHU and BARHOUMA [5].

Table I

Number and percentage of wild bird eggs containing HI antibodies to variants
The eggs were collected in 1978, at different sampling sites

Wild bird species	Date of collection	No. of eggs	Number/percentage of eggs		
			A/Victoria/75	A/Texas/77	A/H1N1/77
			variants		subtype
Common tern	22 May	196	N	45/22.9	N
	1978	148	N	21/14.1	31/20.9
		191	30/15.7	22/11.4	41/21.4
	Total	535	30/5.6	88/16.4	72/13.4
Black-headed gull	22 May	90	10/11.1	34/37.7	N
	1978	66	18/27.2	17/25.7	16/24.2
	Total	156	28/17.9	51/32.0	16/10.2

N = negative

Results

The results of yolk antibody assays differed between species and sampling sites. The incidence of infection with Texas/77 and Victoria/75 variants was two- and threefold, respectively, among the black-headed gulls which had arrived from the Balcan, Mediterranean and Black Sea regions compared with the incidence among the common terns, which had returned from Africa. The subtype A/HlNl/77 showed a roughly equal incidence in both species. The avian influenza virus subtype Hav1 with 7.6% was comparatively frequent among black-headed gulls. In the common tern population the subtype Hav7 showed a comparable incidence, while yolk antibodies to the other subtypes were found in 1.0–2.0% of the eggs studied (Table I).

The yolk antibodies differed between marshlands within the same pond farm. In two peat march islets the tern eggs contained antibodies exclusively to Texas/77, while the eggs collected in a third, not too distant, march islet, also contained antibodies to subtype HlNl/77. In a fourth sampling area, at the south-western shore of the pond, HI antibodies to Victoria/75, Texas/77 and subtype HlNl/77 were demonstrated in the egg yolks. The black-headed gulls, which not infrequently nest in the same community with common terns, transmitted through the egg antibodies to Victoria/75 and Texas/77 in all sampling areas, usually at higher proportions than the terns. Only in a single gull group was the occurrence of antibodies similar to that established in the tern population.

The yolk titres of HI antibodies to the above A/H3N2 variants and to subtype A/H1N1/77 ranged from 1 : 20 to 1 : 160 in the eggs of both species. The mean titres of yolk antibodies to Texas/77 did not notably differ between

A/Victoria/75 and A/Texas/77, subtype A/H1N1/77, and certain influenzavirus subtypes of peat marsh islets belonging to a pond farm

containing HI antibodies to					
Hav1	Hav3	Hav4	Hav5	Hav6	Hav7
avian subtypes					
N	N	2/1.0	3/1.5	N	9/4.5
N	N	N	N	2/1.3	11/5.6
6/3.1	N	4/2.0	N	2/1.0	21/17.1
6/1.1	N	6/1.1	3/0.9	4/0.7	41/7.6
2/2.2	4/4.4	N	3/3.3	7/7.7	6/6.6
10/15.1	N	N	N	N	N
12/7.6	4/2.5	N	3/1.9	7/4.5	6/3.8

Table II

HI titre of yolk antibodies to A/Victoria/75, A/Texas/77, and subtype A/H1N1/77 in the wild bird eggs shown in Table I

Wild bird species	Date of collection	No. of eggs	HI antibody titres to		
			A/Victoria/75	A/Texas/77	A/H1N1/77
			variants		subtype
Common tern	22 May 1978	196	1 : 10 (5)	1 : 20 (26) 1 : 40 (11) 1 : 80 (6) 1 : 160 (2)	N
		148	1 : 10 (3)	1 : 20 (8) 1 : 40 (9) 1 : 80 (3) 1 : 160 (1)	1 : 20 (11) 1 : 40 (8) 1 : 80 (9) 1 : 160 (3)
		191	1 : 20 (11) 1 : 40 (8) 1 : 80 (9) 1 : 160 (2)	1 : 20 (10) 1 : 40 (7) 1 : 80 (4) 1 : 160 (1)	1 : 20 (9) 1 : 40 (9) 1 : 80 (18) 1 : 160 (5)
Black-headed gull	22 May 1978	90	1 : 20 (6) 1 : 40 (2) 1 : 80 (2)	1 : 20 (7) 1 : 40 (16) 1 : 80 (9) 1 : 160 (2)	N
		66	1 : 20 (3) 1 : 40 (6) 1 : 80 (4) 1 : 160 (5)	1 : 20 (6) 1 : 40 (7) 1 : 80 (3) 1 : 160 (1)	1 : 20 (11) 1 : 40 (4) 1 : 80 (1)

Figures in brackets = number of eggs
N = negative

Table III

Number and percentage of wild bird eggs containing HI antibodies to variants A/Victoria/75 and
The eggs were collected in the same nesting

Wild bird species	Date of collection	No. of eggs	Number/percentage of eggs		
			A/Victoria/75	A/Texas/77	A/H1N1/77
			variants		subtype
Common tern	6 May—21 June 1977	144	17/11.8	—	—
	22 May 1978	535	30/5.6	88/16.4	72/13.4
Black-headed gull	6 May—21 June 1977	129	10/7.7	—	—
	22 May 1978	156	28/17.9	51/32.0	16/10.2

— = not tested; N = negative

gulls and terns, but those of antibodies to Victoria/75 were considerably higher in part of the gull eggs. At the same time, the mean level of antibodies to H1N1/77 was significantly higher in the tern than in the gull eggs (Table II).

Yolk antibody studies conducted in the same sampling area in two subsequent hatching periods, in May, 1977, and 1978, revealed considerable inter-species differences in respect of influenza virus infection (Table III). In May, 1977, the incidence of Victoria/75 was assessed as 12 and 8% among the terns and gulls, respectively, while by May, 1978, it dropped to half among the former, but rose to more than twofold among the latter, relative to the previous year. At the same time, the occurrence of Texas/77 was twice as high among the gulls than among the terns. The subtype H1N1/77 showed roughly the same incidence in both species. Infection by avian influenza virus was dissimilar in the two hatching periods. In 1978, HI antibodies to subtype Hav1 (fowl plague virus) were demonstrated in 7.6 and 1.1% of gull and tern eggs, respectively, although in 1977 no indication was found of the presence of this agent among the gulls. Both species had considerable proportions of yolk antibodies to Hav7 in both hatching periods, although the gulls showed a 75% decrease of incidence 1977 to 1978. A low percentage of the yolks also contained HI antibodies to other Hav-subtypes.

In the pond farm used as sampling area, 11 mallards built their nests near to those of the predominant gull and tern population. A total of 54 mallard eggs was examined for yolk antibodies to the variants Victoria/75 and Texas/77 and subtype Hav1 avian influenza virus with negative results. Neither were such antibodies demonstrable in 48 eggs of captive mallards kept at the river Tisza.

Three months before the onset of the human epidemic due to Texas/77, HI antibodies to this agent had been demonstrated in the titre range of

A/Texas/77, subtype A/H1N1/77 and certain avian influenza virus subtypes in two subsequent years area in both breeding seasons

containing HI antibodies to					
Hav1	Hav3	Hav4	Hav5	Hav6	Hav7
avian subtypes					
N 6/1.1	N N	N 6/1.1	2/1.3 4/0.7	N 5/0.9	10/6.9 41/7.6
N 12/7.6	N 4/2.5	N N	N 3/1.9	N 7/4.5	20/15.5 6/3.8

1 : 48–1 : 256 in 19 of 20 serum samples derived from 5–11 months old SPF (specific pathogen free) chickens kept in the bird unit of a plant manufacturing biological preparations. The same birds had no antibodies to the IR strain of the variant Victoria/75. Two to three months old chickens, hatched three months after the Texas/77 and the H1N1/77 epidemic had subsided, and reared in complete isolation in two different premises, showed a 71–86% incidence of HI, VN and SRH antibodies to A/H1N1/29BP/78 influenza virus at titres ranging from 1 : 24 to 1 : 128.

Serological screening of a large turkey flock kept in a farm near the river Tisza during the human influenza virus epidemic in March, 1978, revealed in HI and SRH tests a 20 and 17.5% incidence of antibodies to the variant Texas/1/77 and to the own isolate of the subtype A/H1N1/29BP/78, respectively. In the goose flock kept in the same farm, 29% of the birds proved to be positive reactors to subtype Hav5 avian influenza virus, but none had antibodies to the above human influenza viruses.

Wild birds shot in the nesting area simultaneously with egg collection, a few days to 2–3 weeks old gull chicks and wild birds shot down in three different regions along the river Tisza were used for isolation experiments. A strain in every respect similar to A/H1N1/77 influenza virus was isolated from the cloaca of a mallard in the third chick embryo passage. Further isolates, corresponding to the A/Texas/25BP/78 strain isolated from humans during the epidemic, were obtained from the cloaca scrapings of two black-headed gulls and from mixed test material collected from the cloaca of six 1–3 weeks old gull chicks. The latter isolations were carried out in embryonated hen's eggs.

Discussion

Serological and virological investigations were carried out in a nesting and hatching area of migrating wild bird with the purpose to obtain more information of their role in the spread of human, mammalian, and avian influenza viruses. During spring, 1977, evidence was presented of infection of black-headed gulls nesting in the marshes around a pond farm with variant Victoria/75 of human influenza virus. The black-headed gulls and the common terns, which established themselves in the same pond farm, returned to Hungary for egg-laying and hatching from Mediterranean areas, where human outbreaks due to Victoria/75 had occurred early in 1977. At return in spring, 1978, both wild bird species had become infected by the variant Texas/77 and subtype A/H1N1/77, for at examination after egg-laying, 32 and 10% of the gulls, and 16 and 13% of the terns, respectively, were found to have transmitted antibodies to the above viruses through the egg. As egg-laying followed soon upon arrival in two to three weeks, the infection of the layers had clearly

taken place before their arrival in Hungary. The gulls had obviously picked up Texas/77 during their winter stay along the Mediterranean Sea, for outbreaks due to the agent did occur there among the population throughout the first quarter of 1978. The common terns which had spent the winter in Africa, may well have become infected by Texas/77 during their return flight *via* the Mediterranean area. Both species had ample chance to pick up H1N1/77, too, as this subtype had been responsible for even more human influenza outbreaks than had Texas/77 in several host countries. In addition to antibodies against Texas/77, a lower percentage of the gull and tern eggs also contained antibodies to Victoria/75; this indicated a booster-like or primary exposure of the layers to the latter variant, which had accounted for outbreaks in the Mediterranean area and in certain parts of Africa up to the beginning of 1978.

In the pond farm marsh islets serving as sampling area, the average laying of both gulls and terns was three eggs. Our yolk antibody assays covered the egg-laying of about 60 black-headed gulls and 180 to 190 common terns, but only of about 10 mallards; in the latter case the limited number of eggs permitted no definite conclusions. Within one nesting community or sampling site, antibodies were generally demonstrable either to Texas/77 alone, or to both Texas/77 and H1N1/77; less often were antibodies present against Victoria/75. This observation indicated that bird groups returning from the same wintering area prefer to establish themselves as one community at the same nesting site.

Another remarkable fact disclosed by the present studies was the presence of serum antibodies to Texas/77 in an isolated group of growing SPF chickens three months before the onset of the Texas/77 epidemic in Hungary. It should be noted that the testing of serum samples from these birds had been prompted by the occurrence of an acute respiratory disease in the flock. Not less remarkable was the detection of serum antibodies to subtype A/H1N1 in two flocks of growing chickens which had been hatched three months after the epidemic. If follows that humans who contracted influenza sporadically long before or after the epidemic period, may spread the infection to domestic poultry. Mass incidence of human influenza virus infections in poultry flocks is thus of prognostic importance, if it occurs in advance of the epidemic, and serves as indicator of the persistence of the virus strain if it occurs after the outbreak has subsided.

Demonstration of yolk antibodies to subtype Hav1 in the gull and tern eggs laid in 1978 suggested that the terns had picked up the Hav1 virus during interruptions of their migration for rest in enzootic foci at the African shore of the Mediterranean Sea and in Egypt, which are on their migration route. The gulls, which migrate to different territories but have a liking for rambling, may have picked up the agent in the resting places. The range of yolk antibodies to the avian influenza viruses classified by haemagglutinating properties as

Hav6 tended to increase from 1977 to 1978, indicating exposure of the layers to an increased number of such strains in the meantime.

Yolk antibody assays revealed that the common terns and black-headed gulls which arrived to Hungary in the previous two springs had become infected by human influenza virus strains during their stay outside the country. Isolation of virus strains in every respect similar to Texas/77 from adult gulls and newly hatched to 1–3 weeks old gull chicks showed that the infection persisted during hatching. The mallards had no antibodies to the human influenza virus strains, but were infected by a virus similar to A/H1N1/77, as shown in isolation experiments. Infection by this strain occurred probably after their return to Hungary but, as many infected mallards failed to show a distinct immune response, it may as well have taken place during their absence from the country. In aggreement with other authors [6, 7] we, too, have found that although anatid birds are as a rule extraordinarily susceptible to influenza viruses, their immune response to primary infection by these is mostly very low. Isolations of Victoria/75 from gulls, and of Hav7 from gulls and terns in 1977, of Texas/77 from gulls and A/H1N1/77 from mallards in 1978, presented firm evidence of the introduction of human and avian influenza virus strains by migrating wild birds. Certain other strains, to which antibodies were found in a low percentage of egg yolks and serum samples (e.g. Hav1), may also have been introduced into the country via these carriers. The nature of the introduced viruses always depended on the infected human and avian populations or environments with which the migrating birds had contact during their winter absence from the country. In the migrating bird populations, which represent several thousand individuals, the virus(es) picked up during migration may persist and spread to other populations, e.g. to pigs [8].

In enzootic foci outbreaks of influenza virus disease among domestic birds have frequently coincided with the return of migrating wild birds to the area [9]. Infection and carriership in wild bird populations are a potential hazard to domestic birds. Influenza virus infection usually takes a clinically inapparent course in wild birds, but transmission to domestic birds usually increases the virulence of the strain, which then elicits symptoms of disease without altering its antigenic pattern [10]. In the nesting areas of the infected wild birds, isolations of influenza virus succeeded chiefly from droppings. Replication takes place in the digestive tract after aerogenic transmission, and the agent is abundantly shed with the faeces. Fluorescence microscopic evidence of intestinal replication of the virus has recently been presented by SLEMONS and EASTERDAY [11].

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SUSCEPTIBILITY OF BIRDS TO TYPE-B INFLUENZA VIRUS

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Among Zoo birds and migrating wild birds examined serologically during an outbreak due to influenza B virus, 4.1% were found to have serum antibodies to the agent. Evidence of avian susceptibility to the agent was emerging from reisolations of the virus and primary and secondary HI antibody response of the birds in subsequent infection experiments performed on adult pheasants and mallards, as well as one-day-old domestic ducklings and chicks. The virus persisted in the pheasants for at least three weeks, and adult pheasants, mallards and domestic ducklings were found to transmit it by contact to part of their susceptible mates.

The spread of type-B influenza virus infection to horses has been known from indirect serological evidence [1] and equine susceptibility was shown by Kasel *et al.* [2] in a group of wild ponies. Spread of influenza B infection to pigs was for the first time demonstrated in Hungary [3–5] in village communities where inhabitants had acquired influenza B infection. Susceptibility of cats to influenza virus B was reported by Lozovaya [6] decades ago. Paniker and Nair [7], too, were able to infect cats and even monkeys with influenza B virus. Canine susceptibility to the agent was demonstrated experimentally by several authors [8–10].

During outbreaks of influenza B in Hungary early in 1977, HI antibodies to the virus were detected in 4.1% of the sera of several Zoo birds (shelduck, ruddy shelduck, raven) and migrating wild birds (black-headed gulls, common, and little terns), as well as in the yolk of gull and tern eggs laid in peat islets belonging to a pond farm. Since the Zoo and the pond farm were both situated in the endemic area, closer investigations were performed into the susceptibility of birds to influenza B virus, in view of the lack of pertinent data in the literature.

Materials and methods

Birds. One-year-old pheasants and mallard ducks reared in captivity, and one-day-old ducklings and chicks, as well as 2–4 weeks old broiler chickens were used in the experiments.

Virus. The standard reference strain B/Hong Kong/8/73 was used for experimental infection and comparative studies.

For re-isolation of influenza B virus samples were taken from the infraorbital cavity and the cloacal mucous membranes of birds, and embryonated eggs pre-incubated for 9—11 days were inoculated intra-amniotically. The eggs were opened as the embryo had died or 3–7 days after inoculation. In the absence of haemagglutination (HA), 3 or 7 consecutive blind passages were carried out.

Sera. Serum samples were obtained for antibody assay from experimentally infected birds of different age groups. Samples obtained from Zoo birds and migrating wild birds, as well as yolks of eggs laid by the latter were used for serological screening.

Serological tests. The haemagglutination-inhibition (HI) test was performed according to the instructions issued by the WHO Expert Committee on Respiratory Virus Diseases [11]. Non-specific inhibitors were eliminated from the sera by inactivation at 56 °C for 30 min and subsequent treatment with KIO_4, and in positive and doubtful cases, with receptor destroying enzyme (RDE, Wellcome Reagents Ltd., London) as well.

The assays for yolk antibodies were carried out by the procedure proposed by CHU and BARHOUMA [12].

Differentiation of IgM from other antibody types. The antibodies appearing during primary and secondary HI antibody response were tested for sensitivity to 2-mercaptoethanol (2-ME; Calbiochem) by the method of HEGE and COLE [13], using 0.1 M 2-ME as test concentration.

Table I

HI antibody response of adult pheasants to B/HK/8/73 influenza virus

Group No.	Pheasants			Virus reisolation		Immune	
		Date of				Exp. infection after	
	Designation	experimental infection	exposure to contact infection	Date	Results	12	20
I	347	16 March 1977		21 March	Positive	1 : 4	1 : 48
	348			21 March	Positive	1 : 32	1 : 128
	346					N	1 : 96
	349					1 : 16	1 : 32
	350					N	1 : 96
	351						
	$K1^0$		16 March 1977		Negative*		
	$K2^0$						
II	352°		6 April 1977	9 May	Negative		
	353°			19 April	Positive		
	354°						
	355°						
	356°						
	357°						
	358°						
	359°						
III	360	28 April 1977		2 May	Positive		
	361			2 May	Negative		
	362						
	363						
	364°		28 April 1977				
	365°						

° in-contact controls

* sinusitis developed; reisolation from sinusal exudate was negative

Results

Detection of antibody to influenza B virus in serum samples from Zoo and migratory birds prompted us to study the avian susceptibility to the agent by experimental infection of adult pheasants and mallards, as well as of day-old domestic ducklings and chicks.

The virus could be reisolated from the upper respiratory mucosa and lungs of 2 female pheasants 5 days after the experimental infection. The other infected pheasants and one in-contact control bird displayed different serum levels of HI antibodies at different samplings. Four birds of this lot died between days 38 and 41 postfection (p.i.). Among eight pheasant cocks (group II) placed with the infected females (group I) 3 weeks p.i. to serve as in-contact controls, influenza B virus was isolated from one (No. 356) at 13

on infection or contact exposure. Results of virus re-isolation

response to				Date of	
contact infection		challenge			
days					
29	40	6 (26 May)	7 (1 July)	emergency slaughter	death
1 : 32	1 : 24	1 : 512		27 May	
1 : 128	1 : 64				6 May
1 : 64	1 : 24				3 May
1 : 8	N				29 April
1 : 96					23 April
				23 March	
				10 May	
				10 May	
				10 May	
					9 May
					19 April
	1 : 24	1 : 192			
				10 May	
	1 : 16	1 : 64			1 June
				2 May	
				2 May	
			1 : 256		
			1 : 512		
			1 : 256		
			1 : 128		

N = Negative

days of exposure, and two developed primary and secondary HI antibody response to the agent. On day 46 p.i., additional six pheasant cocks (group III), four infected and two uninfected were placed with the united groups I and II. Four days later influenza B virus was reisolated from one infected group III cock, and on challenge which followed 64 days after infection, viz. exposure, all group III cocks developed a secondary HI antibody response (Table I). Among a total of 22 pheasants included in this experiment, seven (32%), which had been in a poorer condition than the rest, died between 13 to 56 days following upon infection or exposure. Figure 1 shows the fluctuations of the HI

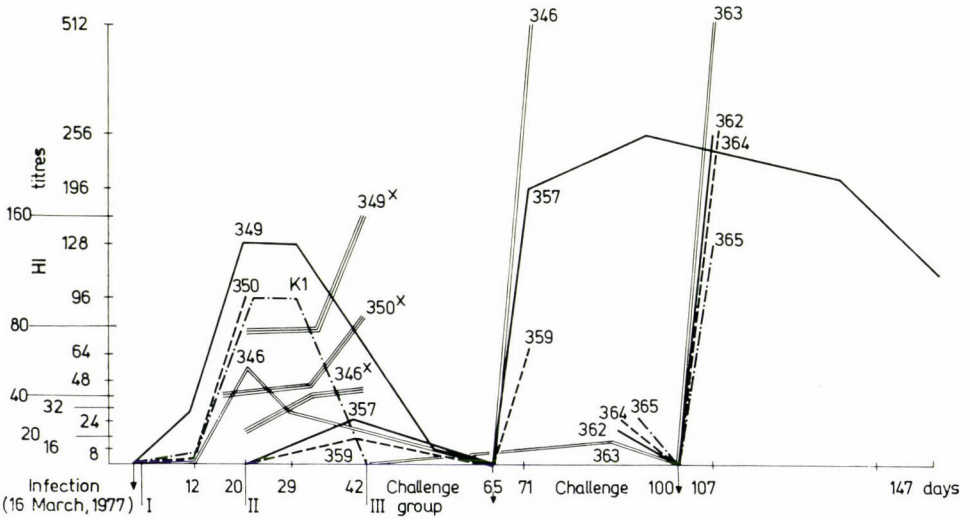

Fig. 1. Haemagglutination inhibiting serum antibody titres of adult pheasants infected with or exposed to, B/Hong Kong /8/73 influenza virus at different times; and HI-titres in egg yolk of infected (group I) females; × = HI-titres in the yolk of eggs laid by the three infected pheasant hens in April

antibody titre of most birds included in the above experiment, and of the yolk titres of HI antibodies found in eggs laid by singly-caged pheasant hens kept in the same room as the experimental birds. Figure 1 also shows the secondary HI antibody response of the experimental birds to challenge, and the firm antibody response developed by one bird (No. 357) in consequence of the booster effect of the challenge dose.

In the next experimental series adult mallard ducks were infected experimentally with the B/HK/8/73 virus strain, and then caged with 6 in-contact control birds. The virus was reisolated 4 days p.i. from the upper respiratory mucosa of one infected mallard (No. 577), and sinusal exudate obtained from this bird was used for infra-orbital infection of two others. The infected mallards and two of the in-contact birds developed primary HI antibody response during

the third to fifth week p.i., and the latter two reacted to challenge at 34 days p.i. by secondary antibody response. The two birds infected with the sinusal exudate also showed secondary HI antibody response on exposure to a booster dose 30 days later (Table II).

Table II

Experimental infection of mallards with influenza virus strain B/HK/8/73

Mallards			Virus reisolation		HI antibody response to		
	Date of				exp. infection contact exposure		challenge (28 July, 1977)
Desig-nation	exp. infection	exposure to contact infection	Date	Results	after days		
					18	33	7 (4 August 1977)
577*	24		28 June	Positive			
578	June				1 : 32	1 : 8	1 : 256
579	1977				1 : 64	1 : 32	1 : 512
1					N	N	1 : 32
2			16 July	Negative	N	.	
3		24 June	11 July	Negative	.	.	
4		1977			N	N	1 : 64
5					N	1 : 16	1 : 128
6					N	1 : 8	1 : 192
580*	28 June				N	N	1 : 128
581*	1977				N	N	1 : 256

* The mallards Nos 580 and 581 were infected intraorbitally with the sinusal exudate of No. 577

N = Negative

· Not tested, previously killed for virus reisolation

In earlier surveys of avian susceptibility to type-A influenza viruses, the anatid birds proved to be most susceptible, especially at 10 to 21 days of age. In view of this, 3 one-day-old ducklings were infected experimentally with influenza B virus and additional 3 one-day-old ducklings were placed with them in the same cage for direct exposure. Serum samples taken from the infected birds at 2, 3 and 4 weeks of age showed HI antibody titres of 1 : 16 to 1 : 64; among the in-contact birds only one showed a measurable serum antibody titre. Growth of the experimental ducklings was inferior to the breed average. On challenge at 70 days p.i., the infected birds developed a secondary HI antibody response; in a single case (No. 575) the booster effect was pronounced at 7 weeks (Fig. 2). Among the in-contact birds one (No. 389) and two infected birds developed a primary and secondary HI antibody response, respectively. Subsequently 25 one-day-old ducks, 10 infected as above and 15 not infected, were caged together. Influenza B virus was reisolated from the upper respiratory mucosa of two infected birds killed 4 days later. At 31 days p.i., 5 infected

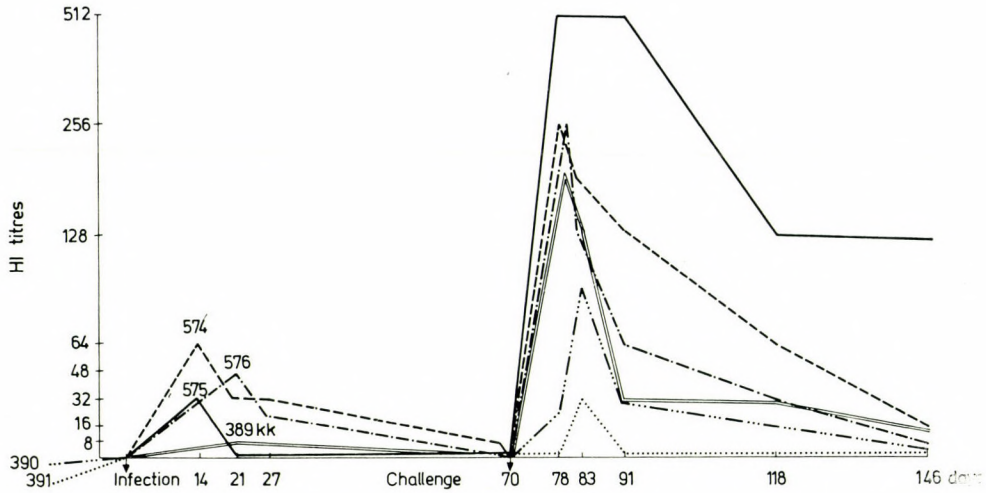

Fig. 2. Fluctuations of HI-antibody levels to B/Hong Kong /8/73 in the sera of three infected and three exposed one-day-old ducklings. Nos 574, 575 and 576 were infected; Nos 389, 390 and 391 were exposed to infection by contact

Table III

HI antibody response of day-old domestic ducklings to B/HK/8/73

Post-infection (1 August 1977) day of		Designation							
		infected							
sampling	challenge	582	583	584	585	586	587	588	589
31		N		4		16		4	
(1.9)			16		8		N		32
	36								
43	5 Sept.	196		128		16		64	
(12.9)			256		512		128		32
67		512		128		32		64	
(6.10)			196		512		64		64
80		32		16		N		16	
(19.10)			64		64		32		N
109		N		8		N		N	
(17.11)			32		32		N		4
	115								
	23 Nov.								
122		128		1024		256		512	
(30.11)			1024		1024		256		128

Explanation: Positive isolation of influenza virus B on 4th day p.i. (5 August 1977) from
N = Negative; — Died previously

ducklings and 2 in-contact control birds had low serum HI antibody levels. Seven days after challenge 6 birds each of the infected (75%) and control groups (40%) developed a secondary HI antibody response (Table III), which persisted over 10 and 7 weeks, respectively. HI antibody response to a second booster dose given at 115 days p.i. also showed that the infection had spread by contact to a substantial part of the control birds.

Domestic chickens of different ages (one-day-old or 2–4 weeks old) were inoculated with the influenza virus strain B/HK/8/73. The agent could be reisolated from the upper airways of a few birds 3–4 days p.i. Among the chicks inoculated infra-orbitally with the sinusal exudate of birds treated with influenza B virus 3 days earlier, one developed a secondary HI antibody response, while the untreated in-contact controls showed a primary response to challenge at 70 days p.i. At 4–8 weeks p.i., the serological HI antibody levels were scarcely measurable in most infected birds, but secondary antibody response to challenge at 45–70 days p.i. still persisted at HI titre levels of 1 : 192 to 1 : 512 more than a month later. The birds exposed to contact infection developed primary antibody response to challenge infection.

influenza virus on infection and exposure by contact

of ducklings														
exposed														
366	367	368	369	370	590	591	592	593	594	595	596	597	598	599
N		N		N		8		N		N		N		N
	N		8		N		4		N		N		4	
N		8		32		128		128		128				
	4		64		256		96		16		8		32	
32		64		64		16		64		32		32		64
	N		64		128		16		32		32		64	
N		16		8		N		N		died		N		N
	N		32		32		8		N	(14.11)	8		32	
N		N		N		N		N		—		N		N
	N		N		N		N		N		8		N	
128		128		256		512		128		—		64		64
	128		1024		1024		256		256		64		256	

the upper respiratory mucosa of ducklings Nos 580 and 581

The primary HI antibody response of birds to infection with the B/HK/8/73 strain was chiefly associated with IgM-type (2-ME sensitive) antibodies, similarly as the response to human influenza A virus. The serological HI antibody levels developed during primary antibody response were markedly depressed by treatment with 0.1 M 2-ME, while the high secondary HI titre levels were scarcely affected by it, if at all. The greater part of the antibodies formed during the secondary antibody response to influenza B virus infection was 2-ME resistant.

Discussion

Pheasants and mallards reared in captivity could be infected with the B/HK/8/73 reference strain of human influenza virus, as verified by re-isolation of the agent from the airways 4–5 days p.i. and demonstration of primary and secondary antibody responses to infection and challenge, respectively. The agent spread by contact to control birds housed with the infected ones, to judge from the secondary HI antibody response of the former to challenge, and from isolation of the virus from in-contact pheasants 13 days after placing them with the infected group at 3 weeks p.i. It follows that the virus persisted in the infected birds for at least 3 weeks. Antibodies were found in the yolk of eggs laid by the infected pheasants at titres similar to the serum levels, but 10–14 days later. The HI antibodies formed during the primary antibody response occasionally persisted for 3–4 weeks, and those formed in response to challenge, for as long as 2–3 months. The pheasants infected experimentally or by contact were retarded in comparison to their healthy contemporaries, and several of them died in 2–8 weeks. The virus could be reisolated from the infected mallards 4 days p.i. and it even could be transferred to two susceptible ones with the sinusal exudate of a diseased bird. The in-contact domestic ducklings and chicks did not show a primary HI antibody response, but part of them developed a long-lasting secondary antibody response to challenge. The infected chicks did not transmit the virus by contact to susceptible ones. The antibodies associated with the primary HI antibody response to influenza B virus were predominantly of the IgM class, while those associated with the boostered secondary antibody response were mostly resistant to 2-ME also in ducks and chicks.

Serological screening of seabirds in the Far-Eastern region of the USSR revealed antibodies to influenza B virus in 1.7% of the examined sera [14]. According to CHERNETSOV *et al.* [15], 7.7% of wild birds migrating and hatching in the Primorye region had been infected by influenza B virus, and SOLOUKHIN *et al.* [16] found a still greater percentage of infection among domestic and wild birds studied in Byelorussia. Among Zoo birds and wild birds examined by

us during outbreaks of influenza B virus infection in Hungary, 4.1% has serum antibodies to the agent. Subsequent experiments performed on adult pheasants and mallard ducks, as well as on one-day-old domestic ducklings and chicks have shown that these species, too, are susceptible to the agent, and the infection of the pheasants may persist for 3 weeks at least. Evidence of transmissibility of the infection by contact was also obtained in all examined species except the domestic chicken, although spread was of a lesser degree compared to that of influenza A virus.

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PURIFICATION AND CHARACTERIZATION OF A DNA-DEPENDENT ATPase FROM BACILLUS CEREUS*

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A DNA-dependent ATPase (molecular weight 71 000) free of nuclease activity has been purified from *Bacillus cereus*. The enzyme shows similar characteristics as the enzyme isolated from *Escherichia coli* and *Bacillus subtilis*. Heat denatured DNA stimulates the rate of ATP hydrolysis to ADP and P_i to an extent about tenfold higher than the native DNA. Double stranded DNA without single stranded regions is not a suitable cofactor for the enzyme. The ATPase is inhibited by adenosine 5'-(β, γ-imino)-diphosphate, while another ATP analogue, adenosine 5'-(β, γ-methylene)-diphosphate has no effect on ATPase activity. K_M for ATP is 0.38 mM, the apparent K_M for nucleotide equivalent DNA is 1.2 μM. Evidence of the unwinding function of the enzyme is presented.

DNA-dependent ATPases containing ATP-dependent DNase activity were described in various bacteria [1–5] and widely accepted as the enzymes of recombination. DNA-dependent ATPases without nucleolytic activity have been detected in *Escherichia coli* [6–9], *Bacillus subtilis* [10], vaccinia virus core [11], in mammalian [12, 13] and plant cells [14]. These ATPases stimulated by single stranded DNA may belong to the multienzyme system of DNA replication, unwinding double stranded DNA for the DNA synthesizing enzymes [15].

An ATP-dependent DNase having DNA stimulated ATPase activity was described earlier in *B. cereus* [5]. Some properties of this ATPase seemed to be similar to the DNA-dependent ATPases devoid of nuclease activity isolated from other bacteria [10, 16]. The question arose whether the DNA-dependent ATPase of *B. cereus* could be isolated in itself, separated from nuclease activity. The present paper describes the purification and main characteristics of a DNA-dependent ATPase from *B. cereus*, specific to single stranded DNA and free of DNase activity.

Materials and methods

Buffer A: 20 mM Tris-HCl pH 7.5, 0.1 mM ethylene-diamine tetraacetic acid, 2 mM 2-mercaptoethanol, 10% v/v glycerol. *Buffer B:* 20 mM Tris–HCl pH 7.5, 0.1 mM ethylene-diamine-tetraacetic acid, 2 mM 2-mercaptoethanol, 30% v/v glycerol. *Buffer C:* 20 mM Tris–

* Work supported by the Hungarian Ministry of Health (4.01.5).

HCl pH 7.5, 1 mM $MgCl_2$, 2 mM 2-mercaptoethanol 30% v/v glycerol. *SSC buffer:* 150 mM NaCl, 15 mM sodium citrate pH 7.0.

^3H—DNA was prepared from *B. cereus* 130 *thy*$^-$ as described earlier [17]. Unlabelled DNA from chicken erythrocytes and yeast RNA were the products of Reanal (Budapest) PM 2 DNA was obtained from Dr. P. MEDVECZKY (Institute of Microbiology, Semmelweis University Medical School), T7 DNA was obtained from Dr. A. FEKETE (Institute of Biophysics, Semmelweis University Medical School). Heat denaturation of DNA was carried out at 100 °C for 10 min in SSC buffer followed by rapid chilling in ice. DNA was sonicated by means of a Sonic 300 dismembrator (Artek) for 1 min in ice bath. UV light irradiated DNA was obtained by irradiation of native DNA by 100 000 erg/mm^2 using a Germicid F lamp (Tungsram).

Single stranded DNA from chicken erythrocytes was linked to CNBr-Sepharose 4B by the method of ARNDT—JOVIN et al. [18] as modified by YOUNG [10].

γ-^{32}P–ATP (200 Ci per mole) was prepared according to POST and SEN [19]. Unlabelled ATP analogues, adenosine 5'-(β, γ-methylene)-diphosphate and adenosine 5'-(β, γ-imino)-diphosphate were from Boehringer.

Protein was determined by the method of LOWRY *et al.* [20]; crystalline bovine serum albumin (Serva) was used as standard.

IgG prepared against DNA helicase II from *E. coli* (50 mg per ml) was the gift of Dr. M. ABDEL-MONEM.

Conditions for nuclease treatment of DNA. Incubation with pancreatic endonuclease (DNase I) in 0.3 ml contained 3 μmoles of Tris–HCl buffer pH 7.5, 1.5 nmole of $MgCl_2$, 48 nmoles of ^{3}H-labelled *B. cereus* DNA and 3 units of pancreatic deoxyribonuclease (Serva). The mixture was incubated at 35 °C for 30 min. The reaction was terminated by heating at 60 °C for 10 min. Acid soluble DNA was removed by dialysis against 200 volumes of SSC buffer. The dialysed material was used as DNase I treated DNA. The control sample was treated similarly except that nuclease was inactivated before addition to the incubation mixture.

S_1 nuclease was used to remove single stranded regions of DNA. The reaction mixture (0.3 ml) contained 10 μmoles of sodium acetate pH 4.7, 50 μmoles of NaCl, 0.3 μmole of $ZnSO_4$, 48 nmoles of ^{3}H-labelled DNA and 20 units of S_1 nuclease (Sigma). The mixture was incubated at 35 °C for 10 min. The reaction was terminated by heat inactivation and dialysed against SSC buffer as described above.

Nuclease assay was carried out as described [5]. The reaction mixture contained in a volume of 0.15 ml, 10 nmoles of nucleotide equivalent ^{3}H–DNA (7×10^3 cpm per nmole), 30 nmoles of ATP, 6 μmoles of $MgCl_2$, 7.5 μmoles of Tris–HCl buffer pH 8.0 and enzyme. A control assay without ATP was always made.

DNA-dependent ATPase assay was carried out by the measurement of $^{32}P_i$ released from γ-^{32}P–ATP. The reaction mixture (0.15 ml) contained 10 nmoles of DNA, 0.5 nmole of γ-^{32}P–ATP ($1–4\times10^5$ cpm per nmole), 150 nmoles of unlabelled ATP, 150 nmoles of $MgCl_2$, 1.5 μmole of Tris–HCl buffer pH 8.0 and enzyme. After incubation at 35 °C for 20 min, the reaction was terminated by addition of 0.2 ml Norit A (Serva) suspension (4% in 0.1 M HCl) and 0.05 ml methanol. After shaking for one minute and standing in ice for 10 min, the charcoal was removed by centrifugation at 4000 *g* and the $^{32}P_i$ remaining in the supernatant was measured in an aqueous solution by means of the Cerenkov effect. Parallel assay without DNA was performed. One unit means the amount of enzyme which degrades 1 nmole of ATP to ADP and P_i in 20 min under the above mentioned conditions.

DNA duplex unwinding assay. The reaction mixture (0.3 ml) contained 10 nmoles ^{3}H–DNA, 10 μmoles Tris–HCl buffer pH 8.8, 1 μmole $CaCl_2$, 30 nmoles ATP, 50 μg bovine serum albumin, 6–30 units of purified DNA-dependent ATPase (Fraction VI) and 2.5 mU micrococcal nuclease (Boehringer). The control reaction did not contain nuclease, ATPase or either of the enzymes. The reaction was incubated at 35 °C for 20 min and then terminated by the addition of 0.2 ml 2 M perchloric acid and 0.1 ml 5 mg per ml bovine serum albumin solution. Undigested DNA was precipitated and after standing at 0 °C for 10 min the sample was centrifuged at 4000 *g* for 10 min; 0.2 ml aliquots from the supernatant were pipetted into counting vials and radioactivity was measured.

Results

Purification of DNA-dependent ATPase. The procedure was similar to that used for purification of ATP-dependent DNase [5]. *B. cereus* NRRL B-569 was grown on Casamine medium [5]. The log phase cells (5×10^8 cells

per ml) were harvested by centrifugation and washed once with 0.5% NaCl–0.5% KCl solution and once with cold Buffer A. Cells were resuspended by adding two ml of Buffer A to each g of cells (wet weight). The cell suspension was stored at –20 °C. All other operations were carried out at 0–4 °C and centrifuged in a Beckman J-21 centrifuge at 12 000 *g* for 20 min unless otherwise noted. A summary of the purification procedure is given in Table I.

Table I

Summary of the purification procedure

Fraction	Volume (ml)	Protein (mg)	kU of activity	Specific activity (kU/mg)	Purification (fold)
I. Extract	23	571	70	0.12	1
II. 40% AS supernatant	22.5	416	72	0.17	1.4
III. 50% AS precipitate	1	24.4	23.8	0.98	8.1
IV. Gel filtration	12	11.0	13.8	1.25	10.4
V. DEAE-cellulose	20	0.8	12.5	15.6	130
VI. DNA-Sepharose	8	0.04	5.4	135	1125

The frozen cell suspension was slowly thawed at 4 °C. Five ml aliquots were disrupted with ten 30 s pulses by a Sonic 300 dismembrator (Artek). The debris was removed by centrifugation. The supernatant was centrifuged at 110 000 *g* for 60 min using a Beckman model L centrifuge. The pellet was discarded, the supernatant retained and dialysed against 200 volumes of Buffer A (Fraction I).

Freshly prepared 10% streptomycin sulphate solution in Buffer A was slowly added by constant stirring over a 20 min period to Fraction I. The volume of the added streptomycin was 1/20 of Fraction I. After mixing for another 20 min the suspension was centrifuged and the pellet containing nucleic acids was discarded. To the supernatant solid pulverised ammonium sulphate (AS) was added slowly over a 20 min period to 40% saturation. After stirring for further 20 min the suspension was centrifuged and the supernatant was saved (Fraction II).

Solid ammonium sulphate was added to Fraction II and brought to 50% saturation as described before, and then centrifuged. The precipitate was resuspended in 1 ml Buffer B and dialysed against 200 ml of the same buffer (Fraction III).

Fraction III was applied to a G-100 column (50×2 cm) previously equilibrated with Buffer B. Active fractions of the gel filtrate were pooled (Fraction IV).

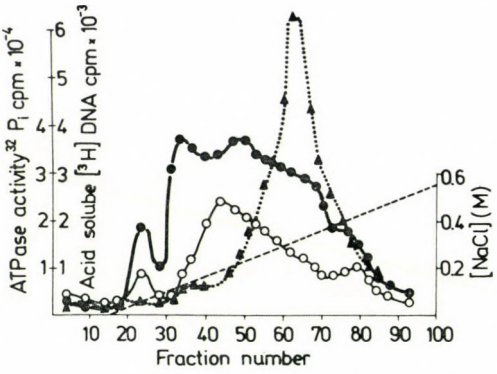

Fig. 1. DEAE-cellulose chromatography of DNA-dependent ATPase. ATPase activity in the presence of ssDNA ●——● and in the absence of DNA ○——○. DNase activity on ssDNA ▲ · · · ▲. NaCl gradient — — —

Fraction IV was applied to a DEAE-cellulose column (12×1.5 cm), equilibrated with Buffer B, washed with 30 ml Buffer B and with a linear gradient (200 ml total volume from 0–0.6 M NaCl in the same buffer (Fig. 1). DNase, specific for denatured DNA eluted in a single peak at 0.32 M NaCl concentration. DNA-dependent ATPase eluted in a broad peak, having DNA independent ATPase activity, too. Fractions (20–40) containing only ATPase activity were combined and dialysed against Buffer C. The dialysed enzyme was concentrated to 20 ml using an Amicon pressure concentrator (Fraction V).

In order to separate DNA-dependent ATPase from the DNA independent one, a column of single stranded DNA-Sepharose (1.5×1 cm) was applied. The column was equilibrated previously with Buffer C. Fraction V was loaded onto the column. Bound ATPase was eluted by a linear gradient (total 40 ml)

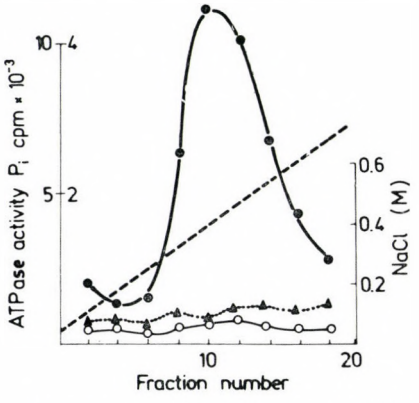

Fig. 2. DNA-Sepharose chromatography of DNA-dependent ATPase. ATPase activity in the presence of ssDNA ●——● and in the absence of it ○——○. DNase activity on ssDNA ▲ · · · ▲. NaCl gradient — — — —

of 0–0.6 M NaCl in Buffer C and 1 ml fractions were collected. DNA-dependent ATPase was eluted at 0.45 M NaCl concentration, DNA independent ATPase did not bind to the column (Fig. 2). DNA-dependent ATPase containing fractions were collected and dialysed against Buffer C (Fraction VI). This material was stored at –20 °C and used for subsequent experiments.

Molecular weight determination. One ml of Fraction VI was applied to the Sephadex G-100 column previously calibrated using bovine serum albumin, pepsin, trypsin and cytochrome C. The ATPase was eluted with Buffer C somewhat earlier than was the bovine serum albumin. The molecular weight of DNA-dependent ATPase corresponds to 71 000 dalton as calculated by the equation of DETERMANN and MICHEL [21].

Properties of the purified enzyme. Assay conditions. The conditions optimal for the catalytic function were tested. The pH in the range from 7.0–9.5 had little influence on the DNA-dependent ATPase activity. The enzyme was stimulated by $MgCl_2$ with an optimum at 1 mM. Addition of $CaCl_2$, $CuCl_2$ and $CoCl_2$ at 1 mM stimulated 85%, 51% and 7%, respectively, of the measured optimal level.

Requirement of ATP. DNA-dependent ATPase activity was measured at different concentrations of ATP in order to determine the maximum rate and substrate requirement of the reaction. A regular saturation curve was obtained. The value of K_M was 0.38 mM (Fig. 3). Competition experiments with two ATP analogues, adenosine 5'-(β,γ-imino)-diphosphate and adenosine 5'-(β,γ-methylene)-diphosphate showed (Fig. 4) that only the imino analogue competed with ATP for the enzyme and caused a 65% inhibition at 0.8 mM analogue concentration. The methylene analogue had no inhibitory effect.

Requirement of DNA. The cofactor specificity of the DNA-dependent ATPase was investigated using different native and denatured nucleic acids

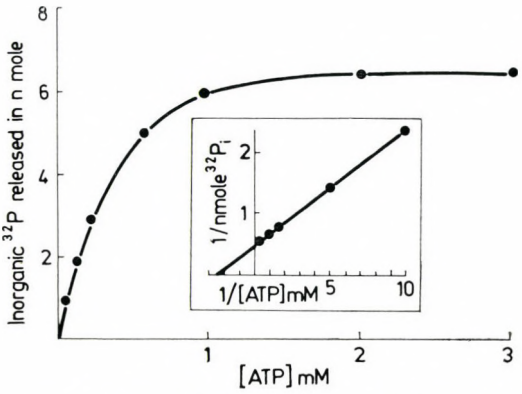

Fig. 3. Effect of ATP concentration on DNA-dependent ATPase activity. The enzyme was assayed under standard conditions with various amounts of γ-^{32}P-labelled ATP. Inset: Lineweaver-Burk plot of the saturation data

(Table II). Linear single stranded DNA either from *B. cereus*, chicken erythrocytes or from T7 phage stimulated a considerably higher phosphohydrolytic cleavage of ATP than the duplex one. Among the native DNA molecules tested, UV light treated DNA did not stimulate more effectively the ATPase activity than the untreated one, indicating that UV induced photoproducts were not the initiators of ATP hydrolysis. Ultrasound produced breaks in the native DNA backbone apparently did not influence the ATPase activity.

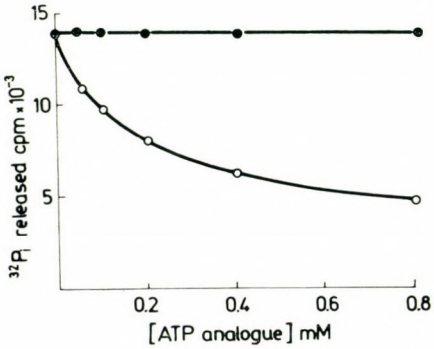

Fig. 4. Inhibition of DNA-dependent ATPase at different concentrations of adenosine 5'-(β, γ-methylene)-diphosphate ●——● and adenosine 5'-(β, γ-imino)-diphosphate ○——○. ATPase activity was measured according to "Methods" with the exception that the reaction mixture contained only γ-^{32}P-ATP (0.5 nmole)

Table II

Effect of nucleic acid cofactors on the DNA-dependent ATPase activity
The reaction micture (0.15 ml) contained 150 nmoles of γ-^{32}P-ATP and 5 nmoles of nucleic acid cofactor

Nucleic acid	P_i nmole/20 min
B. cereus duplex DNA	2.8
B. cereus duplex DNA UV light treated	2.9
B. cereus duplex DNA ultrasound sheared	2.0
B. cereus duplex DNA DNase I treated	9.1
B. cereus duplex DNA S_1 nuclease treated	0.82
B. cereus single stranded DNA	15.8
T7 duplex DNA	1.9
T7 single stranded DNA	21.5
PM 2	0.53
Chicken duplex DNA	2.8
Chicken single stranded DNA	14.7
Yeast RNA	0.45

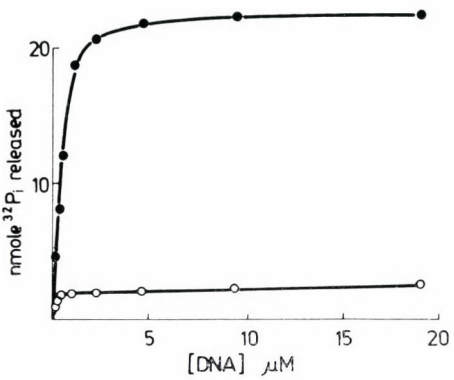

Fig. 5. Effect of ATPase activity of native ○——○ and heat denatured DNA ●——●. Standard ATPase reaction was carried out at various concentrations of T7 DNA

DNase I that resulted in olygodesoxynucleotides with 5'-phosphate terminals increased the ATPase activity about three-fold. On the other hand, S_1 endonuclease that degraded any single stranded regions to mononucleotides decreased the ATPase activity. Circular duplex of PM 2 and RNA were inactive as cofactors.

The DNA-dependent ATPase as a function of double and single stranded T7 DNA showed a regular saturation curve (Fig. 5). The half saturation concentration was 1.2 μM for the single stranded DNA.

DNA unwinding activity. The strand separation effect of the enzyme was followed by measuring the acid soluble DNA released by micrococcal nuclease — a 3'-oligonucleotidohydrolase preferential to single stranded DNA — in the presence and in the absence of DNA-dependent ATPase from *B. cereus*. Native and heat denatured DNA were used as substrate (Table III). The ATPase had

Table III

Stimulatory effect of DNA-dependent ATPase on micrococcal nuclease activity
Unwinding was measured as described in "Methods"

Micrococcal nuclease (mU)	ATPase (units)	Acid soluble material released from	
		native DNA	denatured DNA
		(nmole)	
—	6	0.006	0.007
—	15	0.005	0.008
—	30	0.009	0.008
2.5	—	1.4	7.4
2.5	6	1.8	7.3
2.5	15	2.5	7.6
2.5	30	2.9	7.4

no measurable DNase activity by itself either on native or heat denatured ^{3}H-DNA. The DNase activity of micrococcal nuclease was stimulated about two-fold in the presence of 30 units of DNA-dependent ATPase on native DNA. The control reaction with denatured DNA did not show DNase stimulation in the presence of DNA-dependent ATPase.

Immunological similarities between the DNA-dependent ATPase from *B. cereus* and *E. coli* were studied by addition of IgG produced against helicase II from *E. coli* to the reaction mixture. Fifty μg of helicase II antiserum caused an approximately 80% inhibition on the DNA-dependent ATPase from *B. cereus*. Control samples failed to influence the ATPase activity. This observation suggested an immunological relationship between the *E. coli* and *B. cereus* DNA-dependent ATPases.

Discussion

Experiments undertaken with a 1100-fold purified DNA-dependent ATPase free of nuclease activity showed that the enzyme from *B. cereus* has similar characteristics as those of *E. coli* [7, 16] and *B. subtilis* [10]. The K_M of ATP is 0.38 mM for the *B. cereus* DNA-dependent ATPase, 0.4 mM for the *B. subtilis* [10] and about 0.15 mM for the *E. coli* enzymes [7, 16]. The *B. cereus* enzyme has a molecular weight of 71 000, similar to the *B. subtilis* [10] and the *E. coli* [7, 9, 16] ATPases. It is inhibited by adenosine 5'-(β,γ-imino)-diphosphate like unwinding ATPases from *E. coli* [16, 22] and its activity is hindered by IgG prepared against helicase II from *E. coli*, also suggesting the relationship between the two enzymes.

Linear single stranded DNA is the most effective cofactor of the enzyme. Among the native DNA molecules tested, UV light treated and ultrasound sheared DNA showed nearly the same weak stimulation of ATPase activity as the untreated duplex DNA. The need for single stranded regions of the *B. cereus* ATPase was demonstrated also by the fact that the stimulatory effect of linear duplex DNA was decreased by S_1 nuclease digestion and increased by DNase I treatment.

The DNA-dependent ATPase from *B. cereus* increased the activity of micrococcal nuclease only on native DNA. The increase was explained by the raised level of single stranded DNA produced by the effect of DNA-dependent ATPase, probably by the unwinding of duplex regions. A similar chain separation was observed with the *E. coli rep* protein [9], with DNA binding protein [23] and with DNA unwinding ATPases [24].

Concerning the biological role of the DNA-dependent ATPase, DNA duplex with single stranded regions seems to be the target molecule of the enzyme. The DNA-dependent ATPase–ATP complex probably binds to single

stranded regions, using the energy of ATP hydrolysis for melting the base pairs and keeping the strands separated. The melting of duplex DNA by means of DNA-dependent ATPase is important not only because it points to the possible function of the enzyme in the replication process, but also gives a possible explication how a DNase, e.g. micrococcal nuclease can be ATP-dependent in cooperation with a DNA-dependent ATPase as demonstrated in Table II. In order to confirm the hypothesis that ATP-dependent DNase activity is the result of a cooperation between DNA-dependent ATPase and a DNase specific to single stranded DNA, further experiments are in progress.

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ENTEROTOXIN PRODUCTION OF YERSINIA ENTEROCOLITICA STRAINS*

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Eighteen *Yersinia enterocolitica* serogroup O3, rhamnose negative strains isolated in Hungary from human enteritis, have been studied for enterotoxin production. Freshly isolated strains cultivated at 25 °C produced heat stable (ST) enterotoxin demonstrable in suck ing mice, whereas strains isolated earlier and maintained in subculture lost their toxin producing capacity. No heat labile enterotoxin (LT) was found in the filtrate of the cultures or in their sonicated lysate. The ST of one strain exerted a dilating effect on the intestinal loop of rabbits. Strains cultured at 37 °C produced no toxin. Toxin production was demonstrable in Syncase medium but not in the modified Sakazaki medium. None of the strains caused keratoconjunctivitis; three strains elicited mild conjunctivitis in guinea pigs.

Yersinia enterocolitica is a frequently isolated enteric pathogen in Hungary. The first strain was isolated by RÉDEY [1] in 1965. According to the latest report of SZITA and SVIDRÓ [2] the organism occurs in every part of the country and more than 2000 strains were isolated between 1969 and 1976. Thus it occurs as the third enteritis-causing pathogen in Hungary after salmonella and shigella [2]. In the pathomechanism of *Y. enterocolitica* besides symptoms referring to endotoxin, the role of the enterotoxin may also be assumed [3, 4]. Serotypes of human origin, especially strains not fermenting rhamnose, were found to produce ST type enterotoxin [4, 5]. The aim of the present study was to determine whether ST or LT production could be found in certain representative strains isolated in Hungary and to determine the conditions necessary for demonstration of the enterotoxigenicity.

Materials and methods

Bacterial strains. Five of the 18 strains studied were isolated at the Institute of Microbiology of University Medical School, Pécs. Further strains were obtained from Dr. A. SVIDRÓ (National Institute of Hygiene, Budapest) and Dr. B. RÉDEY (Public Health Station, Veszprém) Part of the strains was isolated between 1974 and 1975 and an earlier isolated laboratory strain, too, was studied. The other strains were fresh isolates. The strains were all rhamnose negative, belonged to serogroup O3 and were isolated from human enteritis cases (Table I).

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Culture media, cultivation. Syncase medium [6]: Casamino acid 2%, yeast extract (Difco) 1%, glucose 0.4%, pH 8.5. Modified Sakazaki medium [7]: Levinthal broth 1000 ml; NaCl 0.35%; mannitol 0.1%; K_2HPO_4 0.368%; KH_2PO_4 0.132%, pH 7.6. Cultivation was performed at 25 °C for 48 hr in a Psycrotherm (New-Brunswick) shaking incubator.

Filtrates. Shake cultures were centrifuged at 7000 rpm for 30 min and the supernatant was filtered through a membrane filter (No. 1121, Göttingen, pore size: 0.45 μm). For the demonstration of heat labile enterotoxin, the cultures were sonicated (MSE, 500 W) before centrifugation.

Molecule filtration. The filtrate of the cultures shaken at 25 °C for 48 hr was dialysed and lyophilized. Two hundred mg of the material were dissolved in 3 ml of 0.005 mole/litre Tris–HCl buffer of 0.001 mole/litre pH 7.6 EDTA content and placed on a 1.5 × 90 cm Sephadex G–100 column. Elution was performed at 20 ml/hr speed, using the above described buffer as eluent. Fractions of 5 ml were collected.

Testing of heat stable (ST) enterotoxin was carried out in 3-day-old suckling mice according to the method of DEAN *et al.* [8] as modified by JACKS and WU [9]. Three mice in each group were inoculated orally with 0.1 ml of the material, then separated from their mothers for 4 hr and subsequently sacrificed. The weight of the intestinal tract was determined and divided by the weight of the body deprived of the intestinal tract. A ratio exceeding 0.083 was considered positive [10].

Isolated rabbit intestinal loop according to DE *et al.* [11] was used with the modification of using 5 cm long intestinal segments. In rabbits of 2–3 kg weight, empty and control loops were prepared and 1 ml of the crude toxin was introduced. Sacrificing the animals 6 hr later the fluid accummulation index $\frac{\text{full-empty intestinal loop weight}}{\text{empty intestinal loop weight}}$ was calculated; a ration exceeding 1 was considered positive.

To demonstrate heat labile enterotoxin (LT), the PF test was used according to EVANS *et al.* [12]. Permeability was determined by introducing intravenously 5% Pontamin Sky blue (Serva, Heidelberg) into white rabbits of 2–3 kg weight, 18 hr after the intracutaneous inoculation, and the result was read after 1 hr. Blueing reaction 5 mm in diameter was the lowest limit of positivity. In some cases the rapid PF effect [13] was studied by introducing the stain 1 hr after the intracutaneous inoculation. For the demonstration of heat labile enterotoxin, the LT effect manifesting itself with an elongation of Chinese hamster ovary cells (CHO [14]) was also studied. SERÉNY's test [15] was applied for estimating invasiveness. The eyes of the guinea pigs were observed for 1 week.

Results

In the first part of the experiments, the *Y. enterocolitica* strains were kept at 25 °C for 48 hr in the incubator and the dilating activity of the filtrates was studied in groups of 3 suckling mice. Syncase medium and the slightly modified Sakazaki medium, which was successfully applied for the demonstration of enterotoxigenicity of different enteral bacteria, were used in these experiments.

Table I summarizes the results. As significant differences were found in ST production between the subcultured and freshly isolated strains, these two groups are presented separately. The culture media applied and the lyophilized crude *Escherichia coli* ST served as control (Table I).

According to Table I, ST production occurred in each of the freshly isolated strains, whereas only one of the subcultured strains had a dilating effect in suckling mice. High index values (as compared with the results obtained with *E. coli* strains) proved that the Syncase medium was suitable and the Sakazaki medium unsuitable for the demonstration of ST entero-

Table I

ST producing capacity of Y. enterocolitica strains

Earlier isolated strains mean index value			Freshly isolated strains mean index value		
Designation of strain	Syncase medium	Sakazaki medium	Designation of strain	Syncase medium	Sakazaki medium
3064	0.054	0.051	29002	*0.150*	.
1087	*0.152*	0.045	6676	*0.090*	.
OKI 134	0.069	0.048	4472	*0.138*	0.072
797	0.063	0.056	31620	*0.127*	0.052
1866	0.079	0.051	6998	*0.121*	.
269	0.066	0.053	52535	*0.087*	.
			3812	*0.101*	.
			48731	*0.132*	.
			45320	*0.155*	.
			62476	*0.176*	.
			61907	*0.162*	0.070
			40476	*0.145*	.
Control: *E. coli* crude ST		0.092			
Culture medium control	0.049	0.045			

Indices printed in italics indicate positive reaction

toxigenicity of *Y. enterocolitica*. Conditions of ST production by a strain were observed and the produced toxin was analysed. This freshly isolated strain No. 4472 was found to produce sufficient ST. Studying the effect of incubation temperature, it was found that while the filtrate of the strain cultured at 25 °C displayed an activity of 0.138 index value, the index was 0.059 and no activity was detected after culturing at 37 °C. The filtrate of the strain retained its activity when kept at 100 °C for 15 min, which confirmed the heat stability of the toxin. The activity of the *Y. enterocolitica* strain No. 4472 and the corresponding preparations was studied in rabbit intestinal loop 6 hr after the inoculation. Results summarized in Table II show that the filtrates and lysates of *Y. enterocolitica* exerted on the isolated rabbit intestinal loop a fluid accumulating effect typical of ST stable at 100 °C. This activity appeared, however, only when culturing was done at 25 °C and it disappeared upon incubation at 37 °C. Live bacteria did show an activity during the short-term experiment. Attempts were made to demonstrate LT activity with filtrates (active) and

Table II

Dilating effect on isolated rabbit intestinal loop of Y. enterocolitica strain. No. 4472 and its filtrates, after 6 hours

Material	Mean index value
Live bacteria*	0.70
Sakazaki (25 °C) filtrate	*1.00*
Syncase (25 °C) filtrate	*1.11*
Ultrasonic lysate	*1.34*
Syncase (37 °C) filtrate	0.30
Syncase (25 °C) filtrate 100 °C 30 min	*1.09*
Positive control: *E. coli* crude ST	*1.36*
Negative controls:	
Sakazaki	0.74
Syncase	0.32

* Bacterial count corresponding to filtrate Indices printed in italics indicate positive reaction

Table III

Fractionation on Sephadex G-100 of the filtrate of Y. enterocolitica strain No. 4472 concentrated by lyophilization. Dilating activity on suckling mice intestine

Fraction		Mean index value
No.	volume, ml	
1–24	1–120	<0.083
25	120–125	0.082
26	125–130	*0.122*
27	130–135	*0.128*
28	135–140	*0.142*
29	140–145	*0.129*
30	145–150	*0.122*
31	150–155	*0.085*
32	155–160	*0.084*
33	160–165	0.067
34	165–170	0.056
35	170–175	0.053

Indices printed in italics indicate positive values

with ultrasonicated lysates. The classical (delayed) PF test and the CHO test gave negative results. The rapid PF activity is not specific for LT as observed also by others [16].

To obtain information on the molecular weight of the *Y. enterocolitica* ST, the filtrate of the strain No. 4472 concentrated with lyophilization was subjected to fractionation on Sephadex G-100. Results are presented in Table III.

The activity began to appear in the 25th fraction (120–125 ml), reached its peak in the 28th fraction (135–140 ml), and was still present in the 32nd fraction (155–160 ml). This result corresponds roughly to the data on the molecular weight of *E. coli* ST, thus it refers to a molecular weight of 10 000.

Finally, tha invasive capacity was studied with Serény's test. Neither of the strains caused keratoconjunctivitis, although a mild conjunctivitis developed upon the effect of a few freshly isolated strains. Data in the literature [17] concerning the enterotoxigenicity of *Y. enterocolitica* emphasize that toxin producing strains rarely ferment rhamnose and salicin. In the present experiments none of the strains fermented rhamnose and only two strains caused salicin fermentation.

Discussion

The present results showed that enterotoxigenicity was not unfrequent with freshly isolated *Y. enterocolitica* strains. The amount of enterotoxin produced was not negligible, as shown by the dilatation index determined in suckling mice. The ST enterotoxin had a characteristic activity in suckling mice, caused an early reaction on the rabbit intestinal loop, and showed a characteristic range of molecular weight. Heat treatment at 100 °C did not influence its activity. Thus the results confirmed the data published [3–5].

No data are available concerning the genetic background on enterotoxin production. Considering the fact that each of the strains isolated by Pai and Mors [5] from clinical cases produced enterotoxin, these authors assumed a chromosomal origin. The observation, however, that the majority of subcultured strains lost their ST producing capacity refers rather to a plasmid information. The question required direct examinations. Determination of the optimum culturing conditions was an essential requirement of the present work. The advantage of the Syncase medium over the more complete Sakazaki medium was striking. The bacterial count yielded by the former medium was by 1 log10 exponent higher.

Optimum growth at 25 °C is another generally observed characteristic of *Y. enterocolitica*. The present experiments revealed a difference in growth as great as 3 log10 exponent between at 25 °C and 37 °C. It is also known that

the Voges–Proskauer reaction [18] and the ciliary movements [19] are positive only at room temperature. According to LEE *et al.* [20] some *Y. enterocolitica* strains of human origin penetrate into HeLa cells only if cultured under 36 °C. CARTER and COLLINS [21] described a relationship between incubation temperature and mouse-virulence. The mechanism of the pathogenic characteristic of *Y. enterocolitica* is still obscure. One may assume that heat stable enterotoxin plays a role in its capacity to cause enteritis, but the question arises whether toxin production occurs *in vivo* at 37 °C. Several data prove [22, 23] that only ST producing *E. coli* strains are capable of causing enteritis.

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CHARACTERIZATION OF CONIDIATION MUTANTS IN TRICHODERMA VIRIDE BY HYPHAL ANASTOMOSIS AND PROTOPLAST FUSION

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Conidiation mutants were isolated from the fungus *Trichoderma viride*, and tested for complementation by both anastomosis and protoplast fusion. They could be grouped into three classes: (1) colour mutants; (2) mutants with reduced conidiation; and (3) nonconidiating mutants. Several mutants of classes (2) and (3) were incapable of anastomosis, but via protoplast fusion they produced heterokaryons suitable for complementation tests.

Colonies of *Trichoderma viride* do not conidiate in the dark, but by means of a short light pulse they can be induced to produce conidia. Various aspects of this process have been studied, but little attention has been paid to the genetic features of conidiation. Investigation of its genetic background would probably contribute to a deeper understanding of this photo-induced morphogenetic system. Mutants defective in conidiation can easily be isolated [1–3] and tested for complementation by anastomosis [2, 3].

In the present study we have isolated both non-conditiating mutants and mutants with normal conidiation but altered colour, and tested them for complementation. On pairing the mutants, we found that not all are able to form heterokaryons. In certain combinations no complementation was observed in mixed cultures, but formation of green wild-type conidia could be restored by fusion of protoplasts.

Materials and methods

Strain. The wild-type *Trichoderma viride* ex S. F. Gray 8–7 was kindly provided by Professor V. BETINA (Bratislava).

Media. Liquid cultivation medium (CzD) was Czapek–Dox with 0.5% yeast extract and 4% glucose. Agar cultivation medium (CzDA) was as CzD but supplemented with 1.5% agar. For isolation of morphological mutants, CzDA was supplemented to contain 2.4% sodium deoxycholate (CzDAD), and in fusion experiments to contain 0.8 M mannitol (CzDAM).

Inducation of mutations. Mutations were induced by UV according to BOJŇANSKÁ *et al.* [1]. All mutants with altered colour, or with reduced or no conidiation were isolated.

Heterokaryon formation by anastomosis. Three methods were employed for heterokaryon production. (1) Agar blocks were cut from colonies of each mutant grown on CzDA, and placed 3–4 cm apart of fresh CzDA plates. The produced colonies were then allowed to intermingle and produce hyphal connections. (2) Suspensions of the spores or mycelium from the mutants to be anastomised were mixed and aliquots from the mixtures were transferred onto CzDA plates. (3) Each mutant was first cultured in 2 ml CzD for 48 hr. Small pieces from

the pad formed were then taken, mixed pairwise in all possible combinations, and suspended in 2 ml fresh CzD. After a further 48 hr incubation, pieces of the mixed pad were transferred to the centre of CzDA plates. The procedures were carried out in the light.

Heterokaryon formation by protoplast fusion. Protoplasts were prepared from young mycelium grown on cellophane sheets, according to the method described by FERENCZY *et al.* [4] for *Aspergillus*. As osmotic stabilizer, 0.8 M mannitol was used. Protoplasts of two mutatnts to be fused were mixed 1:1 and sedimented by centrifugation (2000 *g*, 10 min). After removal of the supernatant, the pellet was resuspended in 2 ml 30% polyethylene glycol (PEG, mol wt 4000) dissolved in 10 mM $CaCl_2$. In PEG, protoplast aggregates of various sizes developed. After 30 min the suspension was mixed into unsolidified CzDAM at 42 °C and transferred as a thin layer to the surface of CzDAM plates. After solidification, the plates were incubated at 23–25 °C for 5 days in the light.

Results and Discussion

Sixty-seven stable conidiation mutants of *T. viride* were isolated and tested for the ability to produce heterokaryons and restore the wild phenotype in the heterokaryotic state.

With respect to the deviations in morphology, the mutants could be grouped into three classes. (1) Mutants with normal conidiation but altered colour; (2) mutants with reduced conidiation; and (3) non-conidiating mutants. In the first group three further subclasses could be distinguished: white, yellow and brown mutants. In groups (2) and (3) no further division was possible, although the defect in conidiation was usually accompanied by changes in colony morphology, changes in growth rate and release of pigments nto the medium.

Table I

Complementation test of Trichoderma viride conidiation mutants by anastomosis and protoplast fusion

Group	Morphology	Anastomosis	Protoplast fusion
1	changed colour (white, yellow or brown)	complementation between mutants of different colours and groups (2) and (3); no complementation between mutants of identical colour	complementation between mutants of different colours and groups (2) and (3); no complementation between mutants of identical colour
2	reduced conidiation	(a) complementation with any other strain (b) no complementation in certain pairings	complementation
3	no conidiation	(a) complementation with any other strain (b) no complementation in certain pairings	complementation

Heterokaryon tests by anastomosis on the mutants revealed that in most pairings the green wild phenotype can be restored (Table I).

In a few mutants belonging to classes (2) and (3), however, a reduced capability for complementation was observed. Green sectors appeared rarely, and only when the third method of production of anastomosis was employed. Moreover, in certain pairings of some mutants no green sector was found, i.e. the restoration to wild type was not complete.

The feature of a green colour to appear in pairings of mutants identical in colour can probably be attributed to mutations occurring at identical loci. Complementation groups in colour mutants of *Trichoderma* have already been found, and a hypothesis was suggested that 4 structural and 1 regulatory genes interact in the production of green conidia [3]. Our findings support the existence of structural genes, but we failed to isolate mutants with regulatory function.

In classes (2) and (3) no complementation groups were found, although some pairings did not result in obvious complementation. The nonappearance of wild morphology was a consequence not of mutations at identical loci but only the lack of anastomosis. By protoplast fusion all mutants of these types could produce heterokaryons with wild-type morphology (Table I). These results are in constrast with previous observations. WEINMAN-GREENSHPAN and GALUN [2] and GREENSHPAN and GALUN [3] found that, both in colour and non-conidiating mutants, anastomosis, and heterokaryosis can be induced between intact mycelia. Our mutants are probably unable to accomplish anatomosis; this can be attributed either to a secondary mutation affecting the parasexual processes, or more likely to a single pleiotropic mutation which interferes with both anastomosis and formation of conidia.

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NEW MECHANISM OF PLASMID CURING BY PSYCHOTROPIC DRUGS

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Methylene blue enhanced the plasmid curing efficiency of chlorpromazine, imipramine and amitriptyline with strains of *Escherichia coli* K12 carrying F-prime *lac* or the resistance factor R-144. In contrast, methylene blue inhibited the elimination of plasmids by acridine orange and ethydium bromide at all concentrations tested. Two metabolic derivatives of chlorpromazine, chlorpromazine sulphoxide and 7.8-dioxochlorpromazine had no plasmid curing effect even in the presence of methylene blue. Amitriptyline, 7,8-dioxochlorpromazine and acridine orange were effective inhibitors of the conjugal transfer of the resistance plasmid, R–144, whilst methylene blue, chlorpromazine sulphoxide, and imipramine had only slight effects. We were therefore unable to demonstrate a simple correlation between curing ability and inhibition of plasmid transfer amongst the psychoactive drugs tested. A mechanism of plasmid curing by surface action of the drugs is suggested as an alternative to direct intercalation of the drugs into plasmid DNA.

In previous studies [1–3] we have shown that several phenothiazines can efficiently eliminate R-factors or an F-prime *lac* plasmid from *Escherichia coli*. At the same time, some compounds having a similar structure have no plasmid curing effect, e.g. methylene blue and chlorpromazine sulphoxide [4–6]. These compounds are all tricyclic and in some cases, at least, almost planar molecules similar in structure to acridines, compounds which have been used to eliminate a variety of bacterial plasmids [4, 7]. Furthermore, acridines and e.g. chlorpromazine are able to intercalate into DNA *in vitro* [8–10]. Consequently, we suggested previously [3, 11] that the mechanism of plasmid curing by phenothiazines might be based on an acridine-like intercalation into DNA *in vivo* which results in differential inhibition of plasmid replication. A similar hypothesis has been put forward by Hahn *et al.* [9, 12], who showed that a number of intercalatory compounds including chlorpromazine, are effective curing agents. Several observations indicate, however, that the mechanism of action of psychoactive drugs on bacteria may differ from that of acridine. Thus, while acridines are mutagenic under certain conditions [13] chlorpromazine is not mutagenic [11, 14]; *lon* mutants of *E. coli* resistant to several psychoactive drugs are sensitive to acridine [11]; imipramine, a non-planar molecule, does not intercalate into DNA [15] but is an effective curing agent [6], and methylene blue, a molecule which does intercalate into DNA *in vitro*, has no curing activity [4, 6]. Finally, curing by imipramine can be enhanced by methylene blue [6], while the dye actually inhibits curing by

acridines [4]. In the present study we sought to confirm this effect with a number of other psychoactive drugs as well as to compare the effect of acridine and tricyclic drugs upon conjugal transfer of plasmids. By taking account of all available data on plasmid curing by phychoactive drugs and in particular the numerous reports that phenothiazines are surface active compounds in both pro- and eukaryotes [16–19], we also suggest an alternative mechanism of plasmid curing to a direct interaction with DNA.

Materials and methods

Chemicals. Imipramine, amitriptyline and chlorpromazine were obtained from EGYT Pharmaceuticals, Budapest; 7,8-dioxochlorpromazine was kindly provided by Professor Dr. A. A. MANIAN, Psychopharmacology Research Branch, Department of Mental Health, Rockville, Maryland, U.S.A. Chlorpromazine sulphoxide was prepared by the method of FISHMAN and GOLDENBURG [20]. Ethidium bromide was obtained from Serva, methylene blue and acridine orange were obtained from Reanal, Budapest.

Bacterial strains. E. coli K12 LE140, *tsx*, *str*, *Δ lac*, *su*$^-$, λ^R, *mal* (F-prime *lac*$^+$ [3]). *E. coli* C600/R–144 *drd*$^+$ (an R-plasmid bearing strain carrying kanamycin resistance, obtained from Dr. B. WILKINS, University of Leicester), was used as donor and *E. coli* K12 W1A2, *azi lac*$^-$, F$^-$ (a laboratory strain [21]) was the recipient in mating experiments.

Culture media. MTY broth and MTY agar were prepared according to ALFÖLDI *et al.* [22]. Eosin methylene blue agar (EMB) was used for detection of *lac*$^-$ colonies [23].

Elimination of the F'lac plasmid was carried out as described by MÁNDI *et al.* [3]. An overnight preculture of *E. coli* K12 LE140 was diluted 10^4 fold and 0.05 ml (about 5×10^3 bacteria) was used to inoculate 5.0 ml MTY broth cultures. Different concentrations (0 to 200 μg ml^{-1}) of test compounds were added and the tubes were incubated at 37 °C for 24 hr. From tubes showing growth, different dilutions were prepared and 0.1 ml samples were plated on EMB agar. The plates were incubated at 37 °C for 24 hr then counted for *lac*$^-$ (plasmidless) and *lac*$^+$ (plasmid carrying) bacterial colonies.

Elimination of R-plasmids. An overnight preculture of *E. coli* C600/R–144 was diluted 10^4 fold and distributed in 0.05 ml volumes (about 10^3 bacteria) into tubes with 5.0 ml MTY broth. Cultures were supplemented with different concentrations of imipramine and 100 μg ml^{-1} methylene blue. The samples were then incubated without shaking at 37 °C for 48 hr. Dilutions of the cultures were then spread in 0.1 ml amounts onto MTY plates. After incubation overnight, the isolated colonies were replicated onto MTY plates containing kanamycin (50 μg ml^{-1}). After incubation at 37 °C for 24 hr, the number of colonies was compared with the number of colonies growing on the master plate. Control cultures were treated in a similar manner with the same concentrations of imipramine but without methylene blue. The R$^-$ bacteria were checked for *thr*, *leu* and *thi* auxotrophy.

Inhibition of R-plasmid transfer. E. coli C600/R–144 was used as donor and *E. coli* K12 W1A2 as recipient. From overnight precultures of the two strains grown without shaking at 37 °C, 100-fold dilutions were prepared in MTY media and incubated at 37 °C for 6 hr, when the absorbance (A_{620}) was 0.4. Donor and recipient cultures were each diluted 10-fold in MTY media and equal volumes mixed and incubated without shaking in the presence of different concentrations of various drugs added before or after mating pair formation as appropriate. Samples taken at various times were diluted 10-fold in saline and vigorously shaken in a Mickle disintegrator for 1 min. Further dilution were made and 0.1 ml were plated onto selective agar plates. MTY agar containing 50 μg ml^{-1} kanamycin plus 500 μg ml^{-1} sodium azide was used for the estimation of kanamycin resistant transconjugants. The number of donors was determined on kanamycin, and the recipients on sodium azide containing MTY agar. The plates were incubated at 37 °C for 48 hr and the colonies were counted.

In some experiments the donor bacteria were pretreated with the appropriate drugs and after 1 min an equal volume of the untreated recipient bacterial suspension was added in order to determine any inhibition of mating pairs. In other experiments the donor and recipient bacteria were first mixed to allow mating pair formation and after 1 min the drugs were added to the system in order to study the inhibition of plasmid transfer.

Results

First of all the synergistic effect of methylene blue was studied on the plasmid curing action of the two well-known intercalating drugs acridine orange and ethidium bromide. Methylene blue completely inhibited the curing effect of acridine orange (Fig. 1), in good agreement with other data [5]. Ethidium bromide (80 μg ml^{-1}) had a weak curing effect on the F'lac plasmid 0–1.8% and methylene blue decreased this value to 0.2–0.5%.

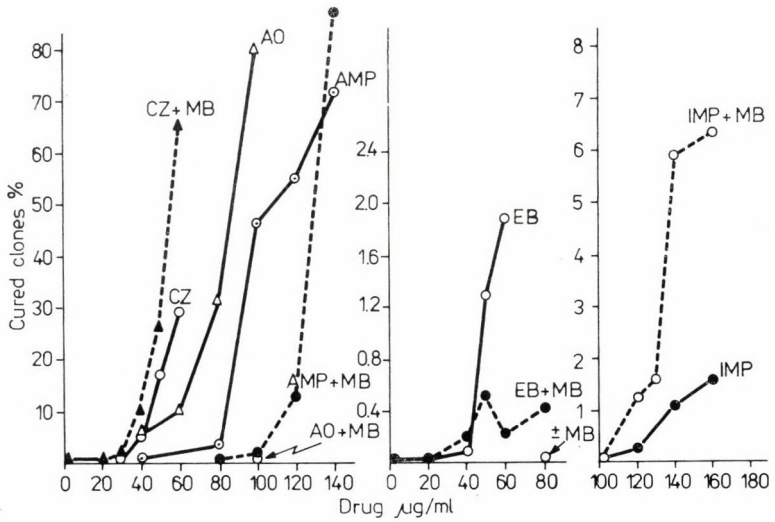

Fig. 1. Effect of methylene blue on the plasmid curing action of psychoactive drugs and some dyes. Plasmid curing action of chlorpromazine (CZ), amitriptyline (AMP), acridine orange (AO), ethidium bromide (EB) as tested on F'lac plasmid. Imipramine (IMP) was tested on R factor R–144 in the presence of 100 μg ml^{-1} methylene blue (MB)

In further experiments, the antidepressant amitriptyline and the tranquillizer chlopromazine were tested for plasmid elimination in the presence of methylene blue. Methylene blue somewhat increased the efficiency of curing of amitriptyline, but the enhancing effect of the dye was more expressed on the curing action of chlorpromazine (Fig. 1). As shown in Fig. 1, the observation that methylene blue potentiated the effect of imipramine in curing F'lac was confirmed with the R-plasmid R-144. On the other hand, methylene blue was not able to alter the effect of inactive metabolites of chlorpromazine e.g. chlorpromazine sulphoxide and 7,8-dioxochlorpromazine. These two compounds had no plasmid curing effect either alone or in the presence of methylene blue. It was interesting that methylene blue increased the plasmid curing activity of the psychotropic drugs, but inhibited the plasmid elimination by acridine and ethidium, in spite of the fact that the dye itself was ineffective

even at high concentration in solid media. On the surface of the solid medium the cell contact or conjugation between plasmid carrying and plasmidless bacteria can be excluded (which may occur in liquid media under usual conditions of curing). In this experiment, of the dilutions of *E. coli* K12 LE140 F'lac a small number of bacteria were seeded onto MTY agar which contained methylene blue at various (0–600 μg ml^{-1}) concentrations. The plates were incubated at 37 °C for 48 hr, then the colonies were checked for lactose fermentation. It was found that none of the colonies had become lac-negative.

Since methylene blue was ineffective as a plasmid curing agent, and it nevertheless exerted an enhancing effect on the plasmid elimination of amitryptyline and chlorpromazine, we have decided to study the nature of the plasmid eliminating effect by other methods. It seemed reasonable to suppose that the inhibition of plasmid transfer may play an important role in the efficiency of curing. Therefore some experiments were done to study the inhibition of plasmid transfer by some psychoactive drugs and methylene blue.

Effect of curing agents on the conjugal transfer of R-144. Since inhibition of the conjugal transfer of plasmid molecules could provide a mechanism for curing by psychoactive compounds or potentiation by methylene blue, the effect of several tricyclic compounds on transfer of the resistance plasmid

Table I

Effect of some tricyclic compounds on R-plasmid transfer

Drug concentration μg ml^{-1}		No. of kanamycin resistant transconjugants ml^{-1}			No. of donor bacteria	
		1 hr	2.5 hr	5 hr	zero	5 hr
Acridine orange	0	2.0×10^5	4.5×10^5	1.2×10^6	1.1×10^8	4.2×10^8
	50	1.0×10^5	1.2×10^4	6.0×10^4		2.0×10^8
	80	5.0×10^2	7.0×10^2	4.0×10^3		1.3×10^7
	100	1.0×10^2	2.0×10^2	2.0×10^2		2.0×10^6
Methylene blue	0	1.6×10^5	2.4×10^5	2.9×10^6	1.3×10^8	3.8×10^8
	50	9.0×10^4	1.7×10^5	1.4×10^6		2.1×10^8
	100	7.0×10^4	2.2×10^5	7.9×10^5		3.1×10^8
	150	2.3×10^4	2.0×10^5	8.7×10^5		3.3×10^8
Imipramine	0	3.0×10^5	2.4×10^6	3.3×10^6	1.3×10^8	6.6×10^8
	50	1.2×10^5	1.1×10^6	2.0×10^6		2.3×10^8
	100	8.0×10^4	4.5×10^5	7.0×10^5		1.7×10^8
	150	3.7×10^4	3.4×10^5	1.0×10^5		1.2×10^8
Amitriptyline	0	4.8×10^5	5.0×10^5	1.2×10^6	1.5×10^8	3.9×10^8
	50	1.0×10^5	2.1×10^5	2.5×10^5		2.0×10^8
	80	1.0×10^4	2.1×10^4	3.6×10^4		1.2×10^8
	100	0	0	0		4.0×10^7
Chlorpromazine-sulphoxide	0	1.4×10^5	5.0×10^5	8.2×10^5	1.2×10^8	3.3×10^8
	150	6.0×10^4	1.5×10^5	2.0×10^5		2.0×10^8

The kanamycin resistant donor strain was pre-treated for 1 min with the indicated drug then mixed with an equal volume of the recipient strain and the number of transconjugants was determined as in Methods. *E. coli* C600/R144 was used as donor

R-144, was examined. Initally, the donor strain was pretreated with various drugs for 1 min before addition of the recipient and the number of transconjugant (kanamycin resistant) clones subsequently determined. As shown in Table I, acridine orange markedly inhibited the transfer of the R-plasmid as found previously by CUZIN and JACOB [24]; imipramine was somewhat less effective, matching its low level of curing, and methylene blue had almost no effect. The efficient curing agent, amitriptyline, appeared completely to inhibit conjugal transfer at a concentration of 100 μg ml^{-1} (Table I).

As the above data show, inhibition of plasmid transfer might have arisen through the inhibition of initial mating pair formation rather than by any direct effect on DNA transfer. Therefore in further experiments, the donor and recipient cells were first mixed and after 1 min (to allow commencement of pair formation) the drugs were added. The data in Table II demonstrate that the addition of acridine orange, amitriptyline, 7.8-dichlorpromazine and to a lesser extent imipramine, still promptly blocked plasmid transfer. Similar results were obtained when the drugs were added up to 15 min after mixing the strains.

Table II

Inhibition of R-plasmid transfer by psychoactive drugs and acridine orange

Drug	Bacteria	No. colony formers (ml^{-1}) min after mixing		
		1	30	60
Control	Donors	2.8×10^8	2.9×10^8	3.3×10^8
	Recipients	3.5×10^8	3.5×10^8	3.8×10^8
	Kanamycin resistant transconjugant	1.2×10^3	2.5×10^4	9.8×10^4
Acridine orange	Donors	2.7×10^8	2.2×10^8	5.9×10^8
	Recipients	3.5×10^8	2.7×10^8	5.0×10^7
(80 μg ml^{-1})	Kanamycin resistant transconjugant	1.0×10^3	2.0×10^3	2.4×10^3
Amitriptyline	Donors	2.5×10^8	2.0×10^8	9.3×10^7
(80 μg ml^{-1})	Recipients	3.0×10^8	1.8×10^8	8.2×10^7
	Kanamycin resistant transconjugants	1.0×10^3	1.2×10^3	2.3×10^3
7,8-Dioxochlor promazine	Donors	2.6×10^8	9.0×10^7	5.0×10^7
(120 μg ml^{-1})	Recipients	3.5×10^8	1.2×10^8	9.0×10^7
	Kanamycin resistant transconjugants	2.3×10^3	1.0×10^3	7.0×10^2
Imipramine	Donors	2.8×10^8	2.8×10^8	9.0×10^7
(300 μg ml^{-1})	Recipients	3.0×10^8	2.0×10^8	7.7×10^7
	Kanamycin resistant transconjugants	2.0×10^3	4.0×10^3	4.2×10^3

Donor and recipient strains were mixed and incubated at 37 °C for 1 min before addition of the drug

Discussion

Two major mechanisms of plasmid elimination from bacteria have described; that acridine or ethidium dyes intercalate into DNA molecules eventually to inhibit replication [4, 8, 9], although it is not clear why this should lead to a differential effect on plasmid replication; curing by sodium dodecyl sulphate (SDS) has been ascribed to the selection of plasmidless clones due to their greater resistance to SDS [25, 26]. We have previously reported that *E. coli* cured of F-prime *lac* by phenolthiazines is not more resistant to the drugs than the plasmid bearing parental strain [6] suggesting that a selection mechanism is not operating. On the other hand, several teams have reported that chlorpromazine intercalates into DNA *in vitro* [9, 15, 27, 28]. It was shown that the drug inhibits DNA polymerase *in vitro* [10, 29], however, unlike acridines, chlorpromazine and other phenothiazines are not mutagenic for bacteria [11, 14]. In addition, as indicated in the introduction, there is a poor correlation between the known curing efficiency of a number of psychoactive drugs and dyes and their apparent ability to intercalate into DNA *in vitro*. We conclude therefore that curing by phenothiazines and other psychoactive tricyclic compounds may not involve intercalation into DNA or indeed any interaction with DNA. Consistent with this are the recent studies of the nature of the surface envelope of *E. coli* and *Salmonella typhi-murium*, (reviewed by NIKAIDO [30]), which indicate that hydrophobic drugs and dyes like gentian violet and methylene blue fail to penetrate the outer membrane of the envelope, although they may bind to it [30–32]. The hydrophobic nature of compounds like chlorpromazine, imipramine and amitriptyline suggest that they will also be excluded by the outer membrane and studies with [^{3}H]-imipramine we failed to detect any active uptake of the drug by *E. coli* B/r strains (unpublished results). Chlorpromazine is known to bind strongly to cell membranes, possibly by intercalation into phospholipid or similar bilayers [19, 33, 34]. Consequently, we suggest that plasmid curing by psychoactive drugs may be due to an action at the bacterial surface, perhaps leading to failure of plasmid molecules to segregate (partition) to daughter bacteria. An alternative possibility, that inhibition of conjugal transfer within a population of plasmid bearing bacteria might contribute an important element in the curing process, appears unlikely since 7,8-dioxochlorpromazine although an effective inhibitor of R factor transfer, has no detectable curing activity. Similarly, the potentiation of chlorpromazine action by methylene blue cannot be ascribed to an effect of the dye on re-infection by conjugal transfer, since the dye alone had little effect upon the transfer process.

The cause of the stimulatory effect of methylene blue on curing by psychoactive drugs remains uncertain but may involve an intermolecular reaction in which an electron is transferred from the drug to the dye, producing

a free radical derivative of the drug which then binds more tightly to its biological target, perhaps in the bacterial surface. These results suggest that inhibition of conjugal transfer is not essential in plasmid elimination.

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NEW HAEMAGGLUTINATING FIMBRIAE ON ESCHERICHIA COLI STRAINS ISOLATED FROM URINE*

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The haemagglutination patterns of 255 urinary *Escherichia coli* isolates were examined with human (A, Rh^+), bovine, chicken and guinea pig erythrocytes in the presence and absence of D-mannose. The strains were divided into four groups according to their haemagglutination properties. About 40% of the isolates agglutinated human red blood cells in the presence of D-mannose. The haemagglutinin of one of these, *E. coli* O18a, c: K^- strain No. 119 was stable, temperature sensitive, did not develop at 18 °C and could be isolated by the methods used for the production of fimbriae. Electron microscopy showed fimbriae on the surface of strain No. 119. An absorbed serum prepared from a derivative cured of haemagglutinating property (No. 119/1) agglutinated all the strains haemagglutinating human erythrocytes in the presence of mannose, but none of those having other haemagglutination patterns. Serologically, the antigen of No. 119 is independent of the K88, K99, "987" and CF I factors and shows some relationship to CF II.

During the past few years, several adhesive fimbriae were identified as important additional virulence factors of enterotoxigenic *Escherichia coli* strains. They play an essential role in the enterotoxic enteropathy of domestic animals [1–3] and man [4, 5]. These fimbrial adhesion factors share numerous common properties, but their antigenic structures and haemagglutination patterns are different. Based on the latter difference EVANS *et al.* [6] developed a haemagglutination typing system which allows to detect fimbriated strains and new adhesive factors. Using their method we often found a haemagglutination pattern among urinary *E. coli* isolates, which was relatively rare among their 823 strains isolated from faeces.

In the present paper we report about the properties of this haemagglutinin.

Materials and methods

Strains. Standard strains carrying adhesion factors were kindly provided by Drs I ØRSKOV and F. ØRSKOV (K88, K99), B. NAGY ("987"), and D. G. EVANS (CF I, CF II).

Media. For quantitative haemagglutination tests or agglutination in factor sera bacteria were grown either on CF medium [7], or on nutrient agar, both being equally suitable.

Sera. Rabbits were hyperimmunized with formalin-treated (0.3%) suspensions of well-fimbriated bacteria. After bleeding, the sera were filtered and preserved by merthiolate (0.01%).

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Factor sera were produced by exhaustive absorption of the diluted (1:10) serum with living and heat-killed suspension of the non-fimbriated derivatives [4].

Haemagglutination tests. Fresh human (A, Rh^+), bovine and guinea-pig erythrocytes were washed three times in PBS pH 7.2 and kept in the original volume in Alsever's solution at 4 °C. Chicken erythrocytes were washed in dextrose–gelatin–veronal buffer (Flow) and kept in the original volume in HEPES–saline–albumin–gelatin buffer (Flow). Working suspensions for slide agglutination tests contained 8–10% erythrocytes in the above buffers with or without 3% D-mannose. Quantitative haemagglutination (HA) tests were made on Takátsy microtitrator plates according to JONES and RUTTER [8]. In HA-inhibition experiments, doubling dilutions of the inactivated sera were incubated at 37 °C with 5×10^7–1×10^8 bacteria/ml for 1 hr, and after adding the red blood cell suspension, incubation was continued for 1 hr at room temperature and overnight at 4 °C. The rocked-tile method of DUGUID *et al.* [9] was used.

Elimination of the haemagglutinin. According to MITCHELL and KENWORTHY [10], about 10^3 cells were inoculated into nutrient broth containing 100 μg/ml ethidium bromide and 0.8 μg/ml mitomycin C. After two passages in this medium, non-fimbriated derivatives were selected with 10% SDS [11].

Isolation of the haemagglutinin was carried out by the methods of STIRM *et al.* [12] and EVANS *et al.* [13]. After cultivating the strain for 24–28 hrs in Roux bottles, cells were harvested in PBS pH 7.2, containing NaN_3 (0.01%). After homogenization at 0 °C for 30 min, bacteria were removed by centrifugation at 5000 *g* for 1 hr at 4 °C and the supernatant was allowed to stand at this temperature for 48–72 hrs. Repeated centrifugation was done with 20 000 *g* for 30 min at 4 °C and the supernatant was acidified with concentrated acetic acid to pH 3.5 and incubated overnight at 4 °C. The resulting precipitate was collected, redissolved in PBS and the whole procedure was repeated three times. After the last precipitation the pellet was washed three times in 0.05 mole/litre acetate buffer pH 4.0 and redissolved in 0.05 mole/litre Tris–HCl buffer pH 8.0.

Electron microscopy was performed by Dr. K. TROMBITÁS (Central Laboratory for Electron Microscopy, University Medical School, Pécs). A drop of the formalinized suspension of agar-grown cells was applied to grids coated with Formwar film and after washing with 0.1 mole/litre KCl it was stained negatively with 1% uranyl acetate. The preparations were examined with a Jeol 100/c type electron microscope.

Results

1. *Haemagglutination patterns of urinary E. coli isolates.* Two hundred and fifty-five *E. coli* strains isolated from patients with urinary tract infection were examined with human (A, Rh^+), bovine, chicken and guinea pig red blood cells in the presence and absence of D-mannose. The collection of strains

Table I

Haemagglutination patterns of 255 E. coli strains isolated from urine

Groups	HA pattern				Number of strains (%)	
	Hu	Bv	Ck	Gp	Hly+	Hly−
I	—	—	—	—	11 (6.79)	40 (43.01)
II	—	—	S	S	62 (38.27)	37 (39.78)..
III	R	—	—	—	56 (34.56)	14 (15.05)
IV	R	—	S	S	33 (20.37)	2 (2.15)
Total					162 (99.99)	93 (99.99)

— no haemagglutination (HA), S = mannose sensitive HA, R = mannose resistant HA
Hu = human (A, Rh^+), Bv = bovine, Ck = chicken Gp = guinea pig erythrocyte

studied consisted of 162 haemolysing (Hly^+) and 93 non-haemolysing (Hly^-) isolates (Table I).

Drawing together groups III and IV, significantly more Hly^+ strains gave mannose resistant haemagglutination (MRHA) with human erythrocytes than with Hly^- ones ($\chi^2 = 34.72$; $P < 0.01\%$). A non-motile, Hly^+, O18a, c : K^-strain, No. 119, belonging to group III was selected for further study.

2. *Properties of the haemagglutinin No. 119.* The haemagglutinating (HA) property of the strain proved to be stable; it was not influenced by passages on nutrient agar plates. Cultivating the bacteria at 18 °C, factor 119 failed to

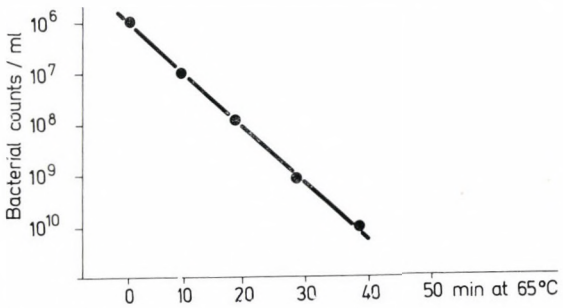


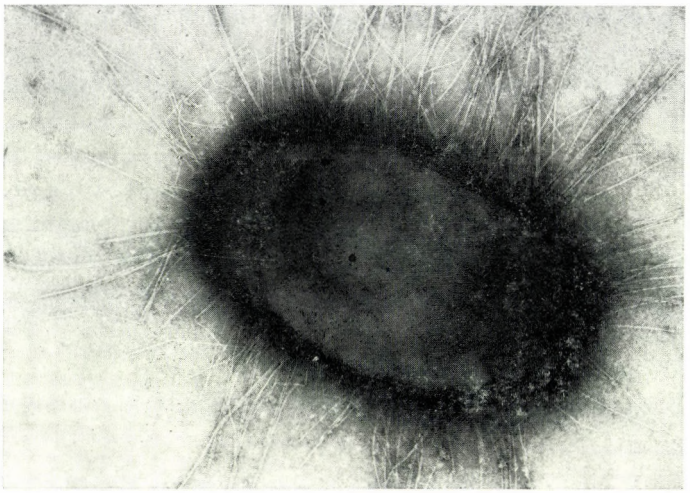
Fig. 1. Effect of heat on the haemagglutinating activity of strain No. 119

develop, and even if the incubation temperature was 20 °C, titres in 119 factor-serum higher than 1 : 80 were not observed, while the corresponding titre of the culture grown at 37 °C was as high as 1 : 6400. Heating of the type strain at 65 °C quickly destroyed its HA activity and after 50 min no HA could be detected (Fig. 1).

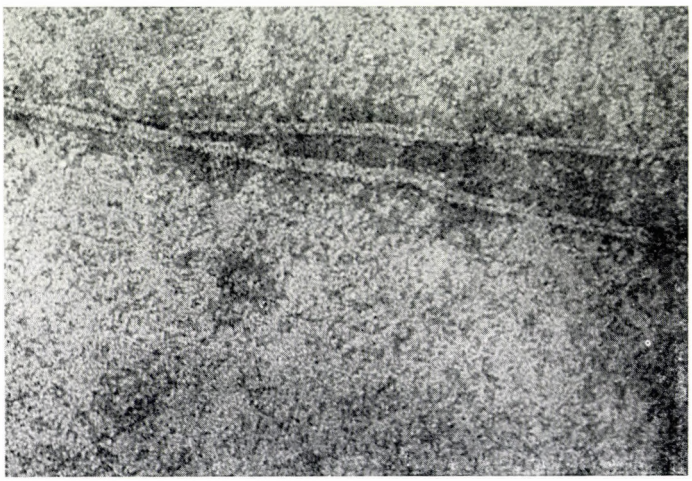
The fact that not all mannose resistant haemagglutinins were associated with fimbriae led us to examine strain No. 119 morphologically. On its surface numerous fimbriae (156 $\pm$ 29/cell), very similar to those of CF I were recognized, while the cured derivative No. 119/1 carried no fimbriae (Fig. 2). Among more than thousand non-fimbriated bacteria of the non-haemagglutinating strain No. 119/1 a few could only be found which had one or two fimbriae. Since these properties resembled the well-known adhesion factors, the question arose whether this haemagglutinin was plasmid-controlled. As spontaneous loss had not been observed, the type strain was cured according to MITCHELL and KENWORTHY [10] and non-fimbriated bacteria were selected in 2.6% by the method of ADACHI *et al.* [11].

The serum prepared against the fimbriated type strain No. 119 was absorbed by the cured derivative No. 119/1. Slide and tube agglutination tests of the 255 urinary *E. coli* isolates gave the following results.

As shown in Table II, strains belonging to groups I and II were not agglutinated in this serum. Members of the other two groups gave titres from



(a)



(b)

(c)

Fig. 2. E. coli (O18a,c:K$^-$:H$^-$) strain No. 119 and its cured derivative 119/1. Negative staining with uranyl acetate. (a) Fimbriated strain No. 119, ×46 800; (b) fimbriae of strain No. 119, ×300 000; (c) cured, non fimbriated derivative (119/1) of strain No. 119, ×46 800

1 : 800 to 1 : 6400. This behaviour of group IV strains supported the idea that these isolates might harbour two kinds of surface structures; the 119-like fimbriae, giving MRHA with human erythrocytes and common pili showing mannose sensitive HA (MSHA) with chicken and guinea pig red blood cells.

Next, the HA inhibiting capacity of this factor-serum was determined and compared to those prepared against the known adhesion factors. Table III summarizes the values obtained in HAI tests and agglutination titres observed with the reference strains and sera. Some cross-relatedness can be seen between the 119 and CF II factors and the findings suggested that the haemagglutinating antigen of strain No. 119 was serologically distinct from other HA^+ fimbriae.

Attempts were made to isolate the haemagglutinin by methods applied earlier for similar purposes [12, 13]. The crude material obtained after repeated precipitations with acetic acid gave MRHA until a dilution of 1 : 1024. The HA activity was temperature sensitive and could be inhibited by 119 factor-serum.

Table II

Agglutination of urinary E. coli isolates in "119" factor-serum

Groups	HA pattern				Agglutinating/all strains tested
	Hu	Bv	Ck	Gp	
I	—	—	—	—	0/51
II	—	—	S	S	0/99
III	R	—	—	—	70/70
IV	R	—	S	S	35/35
Total					105/255

For abbreviations see Table I

Table III

Haemagglutination inhibition and tube agglutination with fimbriated strains and factor-sera

Anti-fimbiral factor-sera	Reciprocal HAItitres	Reciprocal agglutination titres, strains					
		K88	K99	CF I.	CF II.	987	119
K88	<20	1280	.	.	.	.	<20
K99	<20	.	2560	.	.	.	<20
CF I	<20	.	.	2560	.	.	160
CF II	80	.	.	.	5120	.	640
987	<20	.	.	.	.	640	<20
119	320	40	<20	40	160	<20	5120

HAI = haemagglutination inhibition

Discussion

The fact that enterotoxinogenic *E. coli* (ETEC) strains do not invade epithelial cells, and that the typical diarrhoea is caused by cholera-like toxin LT (and ST), led to the discovery of the so-called adhesion factors. These are responsible for the host-specificity of ETEC and their presence is essential for the manifestation of the disease [14, 15]. Thus, in the case of *E. coli* isolated from faeces, the known $MRHA^+$ fimbriae (and 987) are thought to be the bases of adherence.

In the case of extraintestinal *E. coli* isolates besides of other factors [16] the common pili are supposed to be important virulence factors, being responsible for adherence. Their adhesion ability, like their haemagglutination, is known to be mannose sensitive [17, 18] and they have been shown to adhere to epithelial and mucosal surfaces. Their important role in adherence to human uroepithelial cells has been demonstrated [19–22].

It seemed interesting to study whether there are any mannose resistant haemagglutinins on urinary *E. coli* strains. Examining the HA properties of 255 isolates, we often found two patterns i.e. MRHA with human erythrocytes with or without MSHA with chicken and guinea pig red blood cells. These patterns were described by Evans *et al.* [6] who found them among faecal isolates in 2.1 and 2.7%, respectively. About 40% of the strains presented here carried these types of haemagglutinins. The fimbriate morphology of the haemagglutinin on type strain No. 119 was proved, and the plasmid-controlled nature of it was supposed.

There are some essential questions which cannot yet be answered. The relationship between serotypes and the presence of 119 fimbriae should be clarified. It is not known whether these fimbriae confer a colonizing ability to the strains possessing them. Work must be continued in this direction using isolated human uroepithelial cells [19] and some kind of animal model.

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SALMONELLA TYPHI R-PLASMIDS IN HUNGARY

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The antibiotic sensitivity of 4095 *Salmonella typhi* strains isolated in Hungary from January, 1974 to June, 1979, was tested. Twelve strains derived from one patient and seven chronic carriers were resistant to antibiotics due to R-plasmids. One of the *S. typhi* strains carried two R-plasmids and the change in the phage type of this strain was caused by one of the plasmids. R-plasmid of the same restrictive property was found in an *Escherichia coli* strain isolated from the faeces of the corresponding carrier. All of the R-plasmids examined were fi⁻, *E. coli* and *Shigella flexneri* phages were restricted by four R-plasmids, *S. typhi* phages were restricted by one. R-plasmids belonged to incompatibility groups I1, I2, W and H.

Chloramphenicol (Cm) was the most important therapeutic advance in the treatment of typhoid fever, and since 1949, *Salmonella typhi* cultures remained, as a rule, sensitive to antibacterial drugs. Thus, in contrast with other pathogenic enterobacteria, only few infections were caused by Cm resistant *S. typhi* [1–5]. An extensive outbreak of typhoid fever was elicited by a Cm resistant strain in Mexico in 1972–1973. R-plasmid which coded not only a high-level resistance to Cm, causing a minimal inhibitory concentration of 150 μg/ml, but also resistance to streptomycin (Sm), tetracycline (Tc) and sulphonamides (Su) [6]. The R-plasmid belonged to a new incompatibility group, designated H1 [7, 8]. A number of foreign visitors to that country were infected, 52 Americans, two British and one Swiss, after returning to their homes [9, 10].

Joó *et al.* [11] compared the biochemical, immunochemical and immunobiological properties of a Cm-resistant *S. typhi* strain isolated during the typhoid fever epidemic in Mexico and those of a Cm-sensitive strain used for preparing vaccines. They found no difference in the chemical composition of the two strains. The Cm-resistant strain contained less Vi antigen and was less virulent in mice and its active and passive mouse-protective ability was lower than that of the Cm-sensitive *S. typhi* strain.

PANIKER and VIMALA [12] described an outbreak of Cm-resistant typhoid fever in India in 1972. The resistance pattern of the strain was the same as of the Mexican one.

BUTLER *et al.* [13–15] reported that four Cm, Tc, Sm, Su resistant, R-factor carrier *S. typhi* strains were isolated from the blood of 8 patients in Saigon, South-Vietnam, in 1971. The R-factor was similar to that isolated in

the Mexican outbreak. The combined use of ampicillin and trimethoprim–sulphamethoxazole seemed to be the best theraphy.

Among 42 strains obtained in different parts of Canada in January to June, 1973, 5 were resistant to Cm, Tc, Sm, Su, but sensitive to ampicillin (Ap) and their phage type was degraded. The strains were isolated from persons who had visited Mexico or from their contacts [16].

In the Netherlands, out of the 142 *S. typhi* strains isolated in 1972 to 1974 two proved to be resistant to tetracycline and one was resistant to Cm, Tc, Ap and kanamycin (Km) [17].

In Roumania, an R-plasmid carrier Sm, Cm, Tc, Ap resistant *S. typhi* strain was first isolated from a patient in 1972 [18].

The spread of drug resistant strains served as a warning to examine the presence of R-plasmids in *S. typhi* strains in Hungary.

Materials and methods

Bacterial strains. We examined 4095 *S. typhi* strains isolated from patients and carriers in Hungary, from January, 1974, to June, 1979. An *E. coli* strain (resistant to Tc) was isolated from a carrier. Recipient strains in crosses: *E. coli* K12 HfrH nal^r; *E. coli* K12 F^+; *E. coli* K12 J5—3 F^- nal^r; standard *S. typhi* strains of phage type A and Ela; *S. flexneri* 232 rif^r (variant X, phage type 3).

Phages. Phage-set for phage typing of *S. typhi* strains obtained from the Enteric Reference Laboratory and Bureau, Central Public Health Laboratory Service, London. Phage-set for phage typing of *E. coli* strains [19]. Phage-set for phage typing of *S. flexneri* strains [20]. Phages Ms2 [21], f2 [22], and Ike [23].

Reference plasmids (designation of strains/designation of plasmids/incompatibility group): 51/pIP 112/I1; 55/pIP 175/I2; 62/RP4/P; 64/pIP 517/C; 72/pIP 72/10.B.O.; 73/TP 116/H; 67/pIP 518/M (Y. A. CHABBERT, Paris); ./RN3/N; ./S-a/W (N. DATTA, London).

Selective media. Bismuth-sulphite agar, eosin methylene blue agar and desoxycholate agar containing antibiotics, were used. Drug concentrations: chloramphenicol (Cm), tetracycline (Tc), kanamycin (Km): 20 μg/ml; streptomycin (Sm), ampicillin (Ap): 30 μg/ml; nalidixic acid (nal): 50 μg/ml; rifampicin (rif): 250 μg/ml.

Crosses were carried out: (1) cross in overnight broth culture: the 2 hr broth cultures of the donor and recipient strains were mixed 1 : 1; after overnight incubation at 37 °C the mixture was diluted, plated in 0.1 ml quantities on selective agar plates and incubated at 37 °C for 16 hr. (2) by plate method [24].

Determination of fi character. R-plasmids were transferred to *E. coli* K12 HfrH nal^r strain, and the R^+ culture was tested for visible lysis with phages Ms2 and f2, by spot test.

Phage restriction. Phage sensitivity of *E. coli* K12 HfrH nal^r R^- and R^+ strains was examined by routine test dilution of the *E. coli* type-phages by spot-test. In the case of reduced lysis (comparing R^+ strains to the R^- ones), the efficiency of plating (e.o.p.) was assessed by the agar layer method. Restriction of *S. typhi* and *S. flexneri* phages was tested by the same method using adequate recipients and phage-sets. Grouping of R-plasmids based on *S. flexneri* phage restriction was described previously [25].

Incompatibility test was carried out according to CHABBERT *et al.* [26].

Results

Antibiotic sensitivity of *S. typhi* strains isolated from patients and carriers in Hungary, from January, 1974, to June, 1979, was tested. Out of the 4095 strains examined, twelve were resistant to antibiotics and resistance was

due to R-plasmid. Eleven strains were isolated from 7 chronic carriers and one strain was isolated from a patient.

The origin of the R-plasmid carrier strains was as follows.

(1) The first phage type examination of the *S. typhi* strain excreted by the first carrier was carried out in 1960, the phage type was Ela. During the next 15 years no change in phage type was observed. A strain resistant to Sm, Cm, Tc, Ap, Su was isolated in 1975, which was untypable by adapted Vi phages. One year later the carrier excreted sensitive *S. typhi* of the original phage type.

(2) The phage type of the strain from the second carrier was Cl since 1964. The carrier stayed in hospital for one week in 1975 and the strain isolated at that time was resistant to Cm, Tc, Ap, while the phage type was unchanged, Cl. After four months the strain lost its antibiotic resistance.

(3) The third carrier was registered since 1959. In repeated examinations the phage type of the strain was A. It changed to Vi negative in 1974 and 1976, and the strain isolated in 1976 was resistant to Tc. In the next year the strain excreted was sensitive to antibiotics and of phage type A.

(4) The strain was isolated from a patient in 1976. It was of phage type A, and resistant to Cm, Tc, Ap. After recovery, no carrier state developed.

(5) The phage type of the strain isolated from a carrier was A-degraded in 1967, and D1-degraded since 1969. The strain became resistant to Tc in 1977, and proved to be resistant in 1978 and 1979.

(6) This person had had typhoid fever in 1965, the strain excreted was of phage type C1. Phage type remained unchanged during the control examinations. In 1977 the strain was resistant to Tc.

(7) The carrier has been registered since 1968. The phage type of the strain excreted was D1, and it changed to Vi negative in 1971. The strains isolated in 1978 and 1979 were Vi negative and resistant to Tc.

(8) The 8th person was a chronic carrier. The first phage type determination was carried out in 1963, the phage type of the strain was F1. The strains isolated in 1978 and 1979 were of phage type B2-degraded and resistant to Sm, Tc.

In the course of examinations the R-plasmids derived from the same persons in repeated isolations proved to be identical, therefore the Tables only demonstrate one strain and plasmid from each person.

Phage restriction by R-plasmids. Transferring antibiotic resistance from an antibiotic resistant *S. typhi* strain isolated from carrier 1 to sensitive *S. typhi* strains of phage type A and Ela, respectively, colonies of two kinds of resistance pattern were obtained: resistant to Sm, Cm, Tc, Ap, and resistant to Tc. There was no change in phage sensitivity of the *S. typhi* strain of phage type A after the acquisition of Sm, Cm, Tc, Ap resistance, but the phage sensitivity became restricted and the phage type changed to untypable after the acquisi-

tion of Tc resistance. The plasmid determining Sm, Cm, Tc, Ap resistance was designated 1a, the one controlling Tc resistance was designated 1b. R-plasmid 1b introduced to the strain of phage type Ela restricted phages E1 to E10, the strain was susceptible only to phages 46, Vi I + IV and O1, 2, 3. Table I presents the relative e.o.p. values of phages A and E1 mediated by R-plasmids 1a and 1b, respectively.

Table I

Relative e.o.p. values of S. typhi phages in a S. typhi strain carrying different R-plasmids

Designation of R-plasmid	Resistance determinants	Relative e.o.p. values of *S. typhi* phages	
		A	E1
1a	Sm, Cm, Tc, Ap	1	1
1b	Tc	0.2×10^{-3}	0.2×10^{-3}

Two R-plasmids were carried by the *S. typhi* strain and phage restriction was due to that one which determined Tc resistance.

An *E. coli* strain isolated from the faecal flora of the corresponding carrier was also examined. An R-plasmid, similar in phage restrictive effect to plasmid 1b, was demonstrated in this *E. coli* strain. The *S. typhi* strain of phage type Ela changed to untypable after acquisition of the *E. coli* plasmid. The presence of R-plasmid of the same restriction type in the faecal flora allows the sugges-

Table II

Relative e.o.p. values of E. coli type-phages in E. coli strain carrying S. typhi R-plasmids

Designation of R-plasmids	Resistance determinants	Relative e.o.p. values of *E. coli* phages		Plasmid types based on restriction of *E. coli* phages
		23	24	
1a	Sm, Cm, Tc, Ap	1	1	RO
1b	Tc	0.2×10^{-3}	0.3×10^{-3}	R28a
2	Cm, Tc, Ap	0.4×10^{-4}	1	R28b
3	Tc	0.3×10^{-3}	1	R28b
4	Cm, Tc, Ap	1	1	RO
5	Tc	1	1	RO
6	Tc	0.2×10^{-3}	1	R28b
7	Tc, Ap	1	1	RO
8	Sm, Tc	1	1	RO

tion that the R-plasmid was transferred from the *E. coli* to the *S. typhi* in the intestines.

No change was observed in the phage type of the *S. typhi* strain of phage type A after the introduction of plasmids 2 to 8. Changes occurring in the phage types of strains 3, 5, 7 and 8 were not mediated by R-plasmids.

Restriction of *E. coli* phages was examined in *E. coli* K12 HfrH strain after R-plasmid transfer. Restriction of phages 23 and 24 was observed. Table II shows the relative e.o.p. values of the two *E. coli* phages and the restriction types of R-plasmids according to *E. coli* phages.

Table III

Characterization of R-plasmids according to phage restriction and incompatibility

Designation of R-plasmids	Resistance determinants	Plasmid type based on restriction of phages		Incompatibility groups
		E. coli	*S. flexneri*	
1a	Sm, Cm, Tc, Ap	RO	RIII	W
1b	Tc	R28a	RV	H
2	Cm, Tc, Ap	R28b	RV	I1
3	Tc	R28b	RI	I2
4	Cm, Tc, Ap	RO	RI	W
5	Tc	RO	RI	I1
6	Tc	R28b	RV	I1
7	Tc, Ap	RO	RI	I1
8	Sm, Tc	RO	RI	I1

Grouping of *S. typhi* R-plasmids on the basis of restriction of *E. coli* and *S. flexneri* type phages is presented in Table III. The plasmids belonged to three restriction types according to both phage sets. Phage restriction was not caused by plasmids of types RO and RI. Table III shows the incompatibility grouping of R-plasmids; the R-plasmids belonged to the incompatibility groups I1, I2, W and H.

R-plasmids 1a and 1b were transferred to *E. coli* K12 F^+ strain. The F^+ strain carrying plasmid 1b lost its sensitivity to phages Ms2 and f2. H plasmids are incompatible with the extrachromosomal F factor [27].

R-plasmids tested in *E. coli* K12 HfrH strain proved to be fi^-. Visible lysis was observed with phage Ike in the strain carrying plasmid 1a.

Neither *E. coli* nor *S. typhi* phage restriction was observed by an R-plasmid isolated from a *S. typhi* strain derived from the Mexican outbreak.

Discussion

Phage types of antibiotic resistant *S. typhi* strains isolated in Hungary were A, C1, B2-degraded, D1-degraded untypable and Vi negative.

Phage types of resistant *S. typhi* strains reported by ANDERSON were as follows. The Mexican strains (1972) were degraded, the strains from India belonged to Vi-phage type D1-N, the strains isolated in South-Vietnam (1972–1974) belonged to types D6, E7, N, 56 and untypable, strains from Thailand were of types 53, D1 and Vi-negative [9].

Among the examined 8 R-plasmid carrier strains, a change in the phage type after R-plasmid acquisition was observed in one case, when the type changed from Ela to untypable.

A similar phenomenon was reported by RUSU *et al.* [28], who isolated an antibiotic sensitive *S. typhi* strain of phage type A-degraded from a patient, and after four days chloramphenicol treatment the strain isolated was resistant to Sm, Cm, Tc, Ap, carried an fi^- R-plasmid and was resistant to all Vi-phages. It was suggested that the change in phage type was due to restriction-modification mediated by R-factor.

TOUCAS [29] described phage-amplification by R-plasmid. A strain of phage type Ela, sensitive to phage Vi VII, xylose positive, after the acquisition of an R-plasmid derived from a Mexican *S. typhi* strain changed to phage type A, non-sensitive to phage Vi VII and xylose-negative.

Earlier we elaborated and employed a typing method for R-plasmids derived from different bacterial species of *Enterobacteriaceae*, in tracing the epidemiological spread of the plasmids. Typing was based on restriction of *E. coli* and *S. flexneri* phages. *S. typhi* R-plasmids belonged to three restriction types according to *E. coli* and *S. flexneri* phage restriction. Subdivision of plasmids by phage-restriction was completed with incompatibility grouping. There was no correlation between restriction types and incompatibility groups. *E. coli* phages were not restricted (RO) by two plasmids of incompatibility group W but they belonged to different restriction types (R III, R I), according to restriction of *S. flexneri* phages. Five R-plasmids belonged to group I1, two of them were of type R28b and R V, and three of them of types RO and R I.

CHABBERT and GERBAUD [30] characterized *S. typhi* R-plasmids derived from Vietnam and France. The properties of R-plasmids isolated in Vietnam were the same as those isolated in the Mexican outbreak: they were fi^-, belonged to incompatibility group H, and determined Sm, Cm, Tc, Su resistance. The strain isolated in France carried an fi^+ R-factor which belonged to incompatibility group F I and was resistant to Sm, Cm, Tc, Ap, Su.

TOUCAS and VIEU [31] used *S. typhi* strains of phage type A and Ela as recipients and studied the changes in phage-type transferring 22 R-plasmids belonging to different incompatibility groups. They observed restriction of

S. typhi type-phages by R-plasmids of groups I1, 10.B.O., N, W and F I. These findings have confirmed our observation that phage restriction was caused by plasmids of incompatibility groups I1 and W.

The strain carrying a group W plasmid was lysed by phage Ike. Though, according to DENNISON and BAUMBEBG [32], multiplication of phage Ike was supported only by those strains which were harbouring plasmids of incompatibility group N, GRANT *et al.* [33] reported on the multiplication of phage Ike in strains carrying R-plasmids of incompatibility group P.

In one case an *E. coli* R-plasmid was isolated from the faecal flora of a chronic carrier. The plasmid possessed the same phage restrictive property as did the *S-typhi* R-plasmid. It was evident that the *S. typhi* strain acquired the R-plasmid *in vivo* in the intestines. It is assumed that the R-plasmids were acquired in the same way as in the case of the other carriers.

A similar explanation was given of R-plasmid acquisition by *S. typhi* strains by BRANDIS and ANDRIES [34]. They reported a typhoid fever outbreak in Baden-Württemberg in 1974, which was caused by *S. typhi*, phage type A. Out of the examined 262 strains two were resistant to tetracycline. Resistance was due to fi$^-$ R-plasmids. The two R-p lasmids had the same restriction pattern, they restricted Vi test-phages T, D and VII. The patients were treated in the same hospital, though not at the same time. It was supposed that the *S. typhi* strains had received the R-plasmids from other Tc-resistant bacteria in the intestines. In consequence, one cannot underestimate the significance of transfer *in vivo*, though in WATANABE's mouse experiments [35] and in SMITH's self-experiments [36] no such transfer was observed or only in a small degree.

Our studies support the warning that it is of great importance to trace the spread of drug resistant *S. typhi* strains in human populations, the occurrence and spread of R-plasmids in enteric bacteria of human and animal origin, the possible transfer of R-plasmids to *S. typhi* and, in consequence, the decreasing efficacy of chloramphenicol and other drugs in the treatment of typhoid fever.

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ALPHA-HAEMOLYSIN: AN ADDITIVE VIRULENCE FACTOR IN ESCHERICHIA COLI*

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Haemolytic *Escherichia coli*, including human intestinal and extraintestinal as well as porcine enterotoxigenic and oedema disease isolates, and *Proteus morganii* strains were studied for their virulence. Hly^+ wild type strains and Hly^+ transconjugants were more virulent than Hly^- derivatives as shown in mice and chick embryos. This enhanced virulence seems to be connected with the ability of diffusible alpha-haemolysin production because clones producing only non-diffusible, beta-haemolysin behaved as non-haemolytic ones. Haemorrhagic lung symptoms and haemoglobinuria were frequently observed after parenteral challenge of mice with alpha-haemolytic clones. Though the Hly^- clone exhibited a high resistance against blood clearance, the number of circulating bacteria was significantly higher in the case of alpha-haemolytic clone. A causal connection between this phenomenon and the leukocidin activity of alpha-haemolysin is suggested.

Escherichia coli strains producing alpha-haemolysin [1] are known to cause fatal haemorrhagic lung oedema soon after intranasal instillation in mouse [2–4]. The haemolysin is heat-labile, antigenic in nature [1], and its molecular weight is in the 10^5 dalton range [2].

As the lung-toxicity is closely related to the haemolytic property, investigations with Hly^+ wild type strains, Hly^- derivatives and Hly^+ transconjugants were carried out to study the role of haemolysin(s) in the virulence of *E. coli* strains.

Materials and methods

Bacterial strains used are listed in Table I.

Experimental animals. BALB/c and CFLP (LATI, Gödöllő, Hungary) and Rapalovo Breeding Farm mice (USSR), Tetra B hybrid stock chick embryos (Baksa, Hungary) and Pearl of Mecsek rabbits (Pécs, Hungary) were used throughout.

Media. Nutrient broth and nutrient agar (Difco), alkaline extract broth as described by Smith [1] and agar plates containing 5% of defibrinated sheep blood were used. In some cases, especially for detecting beta-haemolysin, erythrocytes were washed three times before adding to agar base. In mating experiments enriched nutrient broth and enriched nutrient agar [6] were used.

Detection of haemolysin. In the first step, blood agar plates were used. Alpha-haemolysin in culture supernatants prepared according to Smith [1] was assayed as described earlier [2]. Ammonium sulphate precipitates of supernatants [7] were dissolved in 1 : 10 of original

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volume in PBS (pH 7.2) and dialysed against PBS (500 : 1 volume) at 4 °C overnight, and tested as simple supernatants. To determine whether the haemolysis on the blood agar plate was due entirely to beta-haemolysin, the overlayer method [8] was used.

Hly⁻ derivatives were prepared either by plasmid curing with actinomycin D [9] or acridine orange [10], or by ethyl-methanesulphonate (EMS) mutagenization [11]. Spontaneous loss of Hly plasmid occurred in some strains.

Transfer of Hly plasmid was performed as described by GOEBEL *et al.* [12]. Strains P673 and P803 were used as donor, and J53 (plasmid free K12 strain) and P673/11 (Hly⁻, lac⁻, Str^R derivative of P673) served as recipients.

Lung test. Mice weighing 10–12 g were infected intranasally with 0.05 ml nutrient broth culture or its dilution under superficial ether anaesthesia. The animals were observed for 48 hr and death and pathomorphological changes were recorded.

Intraperitoneal and intravenous inoculation. Mice weighing 20–23 g were injected intraperitoneally or intravenously with 0.5 ml volumes of serial dilutions of bacterial suspensions. The suspensions were prepared as follows. Nutrient broth cultures shaken overnight were washed twice and reconstituted to the original volume in PBS. The number of colony forming units was determined by plating appropriate dilutions. The animals were observed for a week and signs of oedema and intravasal haemolysis were searched for by autopsy.

Chick embryo test. Ten days old chick embryos were infected through the allantois membrane with 0.1 ml volumes of serial dilutions of bacterial suspensions prepared as described above. Death was recorded 24 and 48 hr after challenge.

Blood clearance test. Mice weighing 20–23 g were given approximately 3×10^8 germs of a Hly⁺ *E. coli* strain and its Hly⁻ derivative. Germ count per ml blood was calculated from samples taken 15 min and later daily from the tail vein.

Leukocidin testing. Granulocytes were separated from the peritoneal cavity of rabbits as described by HIRSCH and CHURCH [13] with the modification that centrifugation was done at 2000 rpm at 4 °C for 10 min. The cells were resuspended in PBS to give a final concentration of approximately 5×10^3 cells/mm³. More than 90% of the cells were granulocytes as revealed by Giemsa staining. One tenth ml ammonium sulphate extract prepared from alkaline extract broth culture supernatants of different derivatives or PBS control were mixed with 0.9 ml of granulocyte suspension and incubated in a water bath at 37 °C for two hours. The proportion of living granulocytes was determined by the trypan blue dye exclusion test using 200 cells pro sample.

Statistical analysis. The LD_{50} estimation method [5] and the Chi square test were applied.

Results

1. *Lung toxicity of alpha-haemolytic E. coli strains.* Five standard enterotoxigenic strains showing alpha-haemolytic character were tested in lung test. All of them caused severe dyspnoea and part of the mice died in consequence of haemorrhagic lung oedema.

As *E. coli* strains causing oedema disease in swine are generally haemolytic, 22 such strains were also investigated. All of them were positive in the mouse lung test.

2. *Hly plasmid elimination and transfer experiments.* Five oedema disease strains (P660, P661, P673, P803 and M-S-15), furthermore the Ent⁺ strain No. 263 as well as strains 281/54 and 18603 of human origin were cured. The altogether 30 Hly⁻ derivatives obtained proved to be negative in the mouse lung test, in agreement with our earlier observation [4].

In the first series of plasmid transfer experiments, the oedema disease strain P673 was used as donor. The cured (actinomycin D) derivative of this strain (P673/1) was EMS mutagenized and a lac⁻ Str^R mutant (P673/11) was

Table I

List of strains

Designation	Antigenic structure	Origin	Note
E. coli K-12 "J53"		N. DATTA	plasmid free
263	08 : K87, K88	B. NAGY	LT^+
2176E8	O138 : K81		ST^+
P99	O141 : K85, K88	H. W. SMITH	LT^+ST^+
P105	O138 : K81		LT^+ST^+
P307	O8 : K87, K88		LT^+ST^+
M-S-15	O139 : K82	B. NAGY	
P866	O8 : K4 : H17	G. SEMJÉN	
P745	O45		
P607			
P124	O138 : K81		
P660	O139 : K82 : H1		
P662			
P661			
P673			
P946	O139 : K82 : H^-		oedema disease strains
P1442			
P803			
P130			
P804			
P809	O139 : K^-		
P1624	O141 : K85ab		
P125			
P820	O141 : K85ac		
P821			
P128			
P123	O147 : K89, K88ac		
P529			
"Kreka"	O4 : K12 : H5		human
281/54	O4		faeces
18603	O4		human urine
P. morganii			
290		S. VÖRÖS	human urine
227			
305/56			human
2380			faeces
2623			

selected. This mutant served as recipient. The recombinants of Hly^+ character were tested for the nature of haemolysin produced. One of the clones producing diffusible haemolysin (P673/111 α) was used in further experiments. A few transconjugants were found to produce only non-diffusible beta-haemolysin. One of them (P673/111 β) was used in animal models.

In further experiments Hly plasmid transfer was carried out using the Hly^+ P673 and P803 strains as donor and the plasmid free *E. coli* K12 (J53) strain as recipient. Transconjugants producing alpha-haemolysin after acquiring Hly plasmid (J53/p673 and J53/p803) were isolated and used in the virulence tests.

3. *Mouse virulence of Hly^+ wild type strains and their different derivatives.* Different strains of mice were infected intravenously with Hly^+ *E. coli* strains or their Hly^- derivatives. In most cases no significant difference could be observed but the Hly^+ clones showed always somewhat higher virulence.

The rather rapid "killing effect" of lethal doses given intravenously was not favourable for the assay of the toxic effect of haemolysin and for the observation of clinical symptoms. Therefore, in further experiments intraperitoneal infection was applied. In these experiments the higher virulence of Hly^+ clones was clearly observable (Table II).

In all cases, independently of the origin of the strains (Kreka and 281/54 of human faecal origin, P99 Ent^+ and P660 pig oedema disease strain), the Hly^+ wild types were about ten times more virulent than their Hly^- counterparts. The summarized data showed significant differences.

Next, the behaviour of Hly^+ oedema disease strain P673 was compared to its Hly^- and Hly^+ derivatives. The wild type strain was strongly positive in the mouse lung screening test but its cured derivative had lost this activity. While the transconjugant P673/111 α reacquired the lung-toxicity, the only beta-haemolysin producer P673/111 β failed to do so. These data together with the results of intraperitoneal mouse infection are summarized in Table III.

Table II

Virulence of some Hly^+ E. coli strains and their Hly^- derivatives in mice infected intraperitoneally

Hly^+ wild-type strains	LD_{50} values* (germs)	Hly^- derivatives (yielded by)	LD_{50} values (germs)	Chi square test ($Hly^+ - Hly^-$)
Kreka (O4 : K12 : H5)	3.0×10^7	Kreka/7 (EMS induction)	3.8×10^8	9.288 $P < 0.01$
P99 (O141 : K85, K88ac)	1.8×10^8	P99/1 (AcD cured)	9.5×10^8	
P660 (O139 : K82 : H1)	9.5×10^7	P660/1 (AcD cured)	9.5×10^8	
281/54 (O4)	9.5×10^7	281/54/1 (AO cured)	3.8×10^8	

AcD = actinomycin D; AO = acridine orange; EMS = ethyl-methane sulphonate
* 4 mice for each dose of the strain tested

Table III

Virulence of E. coli strain P673 and its derivatives in mice infected intraperitoneally and intranasally

P673 and its derivatives	LD_{50} values***	Chi square test; as compared to the wild-type	Lung test (Rapalovo mice) survivor/ infected	Mean survival time (hr)
P673 wild-type Hly$^+$ (O139 : K82 : H1)	5.3×10^8	.	2/10	2.0
P673/1 Hly$^-$ cured	3.9×10^9	8.571 $P < 0.01$	9/9	.
P673/111 α Hly$^+$ transconjugant*	5.1×10^8	0.050 $P \sim 0.80$	0/10	1.4
P673/111 β Hly$^+$ transconjugant**	3.3×10^9	4.285 $P < 0.05$	9/9	.

* Transconjugant, carrying a plasmid (p673 α) responsible for an alpha-type haemolysin
** Transconjugant, carrying a plasmid (p673 β) responsible for a beta-type haemolysin
*** 10 CFLP mice injected intraperitoneally for each dose of the strain tested

Data concerning intraperitoneal mouse virulence showed a significant decrease of virulence after the loss of Hly plasmid ($P < 0.01$) and its perfect recovery when the Hly plasmid coding alpha-haemolysin was retransferred (the probability of an equivalent virulence with the wild type is statistically about 80%). The transconjugant carrying only plasmid coding beta-haemolysin showed a virulence similar to that of the cured Hly$^-$ derivative.

The Hly$^+$ transconjugants of the *E. coli* K12 (J53) strain (J53/p673, J53/p803) elicited a positive response in the lung test only with undiluted shaken cultures. Their intraperitoneal LD_{50} value showed only a moderate decrease as compared to the Hly$^-$ recipient. The low virulence of these strongly haemolytic derivatives may be due to their R like surface character.

4. *Clinical symptoms observed in the mouse model.* Blood tinged foamy fluid could be detected in the nostrils of some mice infected intraperitoneally or intravenously. These animals had haemorrhagic lung oedema as revealed at necropsy. Using the above routes of infection, haemoglobinuria was a frequent symptom in mice given lethal doses. Toxic symptoms were observed only in cases of Hly$^+$ (alpha) clones regardless of being wild type (P673) or transconjugants (J53/p673), J53/p803, P673/111 α), but never with Hly$^-$ or only beta-haemolysin producing derivatives.

The rapid course of the infection, causing death within a few hours, did not permit the development of haemoglobinuria, while doses leading to death between 5–24 hrs after infection caused haemoglobinuria in most of the animals.

Table IV

Blood clearance of Hly$^+$ E. coli Kreka strain and its Hly$^-$ derivative in mice given a sublethal dose intravenously

Time	Number of germs per ml blood	
	Kreka (Hly+)*	Kreka/7 (Hly−)**
15 min	5.6×10^5	6.4×10^5
1 day	8.8×10^{8}***	7.6×10^6
2 days	2.6×10^6	1.6×10^5
4 days	2.3×10^6	1.1×10^3
6 days	1.7×10^8	9.6×10^4
8 days	2.8×10^{6}***	2.8×10^4

* Inoculated with 3.4×10^8 germs
** Inoculated with 2.5×10^8 germs
*** One mouse died

Though the morphological changes in some organs (lung), and the intravasal haemolysis suggested a connection between the higher mouse virulence and alpha-haemolysin production, another approach of the pathomechanism was also taken into consideration. Therefore, the resistance of Hly$^+$ and Hly$^-$ bacteria *in vivo* by means of the blood clearance test was examined (Table IV).

Though Hly$^-$ bacteria were present in the blood of mice during the whole period of observation, the number of Hly$^+$ bacteria was higher at least by one and maximally by three log10 exponents. This observation suggested a leukocidin-like effect of alpha-haemolysin, being analogous to the effect of haemolysins produced by other bacteria [14].

Table V

Leukocidic effect of alpha- and beta-haemolysins of E. coli strain P673

Source of preparation	Percentage of surviving leukocytes	
	after 60 min	after 120 min
P673/111 α	89.0	64.5
P673/111 β	96.0	87.5
P673/1 (Hly$^-$)	97.0	97.5
	Chi square test between alpha and beta-haemolysins	
	$\chi^2 = 29.000$	
	$P < 0.001$	

Experiments with rabbit peritoneal leukocytes showed a moderate but significant leukocidic activity of alpha-haemolysin as compared to the beta one (Table V).

5. *Virulence studies on chick embryos.* As ten-day-old chick embryos are known to be very sensitive to bacterial infections, we hoped that this model would show greater differences in virulence between the Hly^+ and Hly^- clones. The results summarized in Table VI supported this assumption.

Table VI

Virulence of Hly^+ and Hly^- clones of E. coli strains in chick embryos injected into the allantoic cavity

Strains (derivatives)	LD_{50} values* (germs)	Relative potency
P673 (Hly^+)	6.3×10^0	1
P673/1 (Hly^-)	2.7×10^5	$\sim5\times10^{-4}$
P673/111 α	1.0×10^1	~1
P673/111 β	3.5×10^6	$\sim5\times10^{-5}$
J53 (Hly^-)	8.2×10^6	1
J53 (p673)	4.6×10^1	$\sim2\times10^5$
Kreka (Hly^+)	4.3×10^0	1
Kreka/7 (Hly^-)	3.6×10^5	$\sim10^{-5}$

* 4 embryos for each dose of the strain tested

These data showed 4–5 log10 exponent differences between the LD_{50} values of Hly^+ wild type or Hly^+ transconjugant strains and Hly^- derivatives or Hly^- wild types. The Hly^+ derivative of K12 strain was also highly virulent in this model.

6. *Enhanced virulence shown in some P. morganii strains.* According to earlier observations, the alpha-haemolysin produced by *P. morganii* strains was antigenically related to the alpha-haemolysin of *E. coli* (unpublished data). In this study a few strains of *P. morganii* were investigated for haemolytic activity and for virulence in the mouse lung test. All haemolytic strains proved to be lung positive while non-haemolytic strains and cured derivatives were negative in this test. One representative Hly^+ strain (No. 227) and its Hly^- derivative were tested in mice infected intravenously and intraperitoneally. Data are shown in Table VII.

The results presented in Table VII show a close similarity to the observations made on *E. coli*. The Hly^+ clone had a higher virulence for mice both intravenously and intraperitoneally. Summarized data show significant differ-

Table VII

Virulence of a Hly$^+$ P. morganii strain 227 and its Hly$^-$ derivative in mice infected intraperitoneally and intravenously

Strain	Infective doses (germs)	Intravenous infection			Intraperitoneal infection		
		survivor/ infected	mean survival time, hr	haemo-globinuria/ infected	Survivor/ infected	mean survival time, hr	haemo-globinuria/ infected
No. 227 (Hly$^+$)	6.1×10^9	0/5	1.25	2/5	0/5	2.4	0/5
	1.2×10^9	0/5	3.8	5/5	0/5	3.8	3/5
	2.4×10^8	1/5	10	2/5	0/5	4.0	2/5
	4.8×10^7	5/5	.	0/5	3/5	24	0/5
	LD_{50}	*1.6×10^8*			*6.1×10^7*		
No. 227/1 (Hly$^-$)	8.3×10^9	1/5	10	0/5	0/5	15.	0/5
	1.6×10^9	3/5	4.0	0/5	5/5	.	0/5
	3.2×10^8	5/5	.	0/5	5/5	.	0/5
	LD_{50}	*4.0×10^9*			*4.7×10^9*		

Chi square test between Hly$^+$ and Hly$^-$ $\chi^2 = 8.988$; $P < 0.01$

ences in this respect. A more rapid course of the infection and the early appearence of haemoglobinuria were also observed.

Discussion

Data presented in this paper demonstrate the enhanced virulence of haemolytic *E. coli* as well as *P. morganii* strains to be closely associated with alpha-haemolysin production. This type of haemolysin proved in most cases and also in the present experiments to be of plasmid (Hly) origin [12, 15, 16]. There is always a possibility that a plasmid genom carries genes coding different extracellular proteins. E.g. the plasmid for coding pesticin I is also coding a coagulase and a fibrinolysin. Therefore, the parallel loss of virulence and the ability to produce pesticin I does not mean the role of pesticin in virulence [17]. Analogous data are available concerning the presence of ColV plasmid and enhanced virulence in *E. coli* [18, 19]. The relationship between alpha-haemolysin production and virulence may be the same. Our data demonstrate that not only the loss or acquiring of Hly plasmid but also mutations altering the haemolytic phenotype lead to changes in virulence. This suggests the identity of the additive virulence factor and of the alpha-haemolysin but further studies are needed to clarify this question.

Smith [1] described the lethal effect of bacterium-free haemolysin in mice. According to Smith and Linggood [20] the enhanced virulence of haemolytic *E. coli* strains is not in correlation with the amount of haemolysin produced. Fried *et al.* [21] observed a decreased nephropathogenicity of a

Hly$^-$ mutant as compared to the Hly$^+$ wild type *E. coli* strain. The fact that many other haemolysins of bacterial origin are more or less important factors of virulence, such as staphylolysin, streptolysin, listeriolysin, the vibriolysins, etc., supports the assumption that the alpha-haemolysin, too, may be involved in the pathogenesis of *E. coli* infections.

In domestic animals the relation of haemolytic character and pathogenicity is the most striking in oedema disease of pigs. The selection of such pathogens is based on the haemolytic character of *E. coli* colonies.

As regards human infections, DUDGEON *et al.* [22] observed a high proportion of haemolytic isolates from urinary tract infections. The incidence of haemolytic *E. coli* among extraintestinal isolates is ranging between 26% [23] and 56.8% [24]. The proportion of haemolytic strains in faecal samples is much lower [1, 25]. The factual role of haemolysin in the pathomechanism of *E. coli* infections of humans or domestic animals is, however, still speculative and needs further investigations. It should be taken into consideration that if the alpha-haemolysin is really a factor of virulence, its role remains additive and is only one factor among the others [18, 26–29].

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NOTE

EFFECT OF CHLORHEXIDINE GLUCONATE ON THE SURVIVAL OF ACID FAST BACTERIA

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Mycobacterium and *Nocardia* species were examined for survival after exposure to chlorhexidine gluconate. In clinical samples *M. smegmatis*, *M. phlei*, *M. marinum*, *M. gordonae*, *M. scrofulaceum*, *M. kansasii*, *M. chelonei* complex, *M. fortuitum*, *M. flavescens*, *M. avium*, *M. xenopi* and *Nocardia* sp. survived pre-treatment with the agent. After exposure of saline suspensions of bacteria to chlorhexidine gluconate, *M. smegmatis*, *M. phlei*, *M. marinum*, *M. gordonae*, *M. pellegrino*, *N. corallina*, *N. rubra* and *Rodochrous gordonae* were not recovered, and *M. fortuitum* and *N. asteroides* grew poorly.

Biological studies on polyguanidines have shown that 1,6-di-4'-chlorophenyldiguanido-hexane (Hibitane®) exerts a selective antibacterial effect [1]. *Mycobacterium tuberculosis* can be recovered after an exposure for 24 hr, whereas other bacteria are killed. Another polyguanidine, chlorhexidine gluconate acts similarly [2]. On the basis of this finding, chlorhexidine gluconate has been introduced for the treatment of clinical samples prior to culturing for *M. tuberculosis* [3]. Hence, it was of interest to examine the effect of the agent on other acid fast bacteria.

Methods. *Mycobacterium* and *Nocardia* species and *Rodochrous gordonae* (Table I) were suspended in physiological saline and adjusted to density grade 1 of the McFarland scale. Four ml of 0.125% aqueous chlorhexidine gluconate solution were mixed to 1 ml bacterial suspension. After shaking by hand, the suspensions were left to stand at 37 °C for 4 hr, then at room temperature for 16 hr. Löwenstein–Jensen media were inoculated with 0.1 ml aliquots, incubated at 37 °C and read at two day intervals for 28 days. Clinical samples of 2 ml volume were mixed with 10 ml 0.125% chlorhexidine gluconate and left to stand at 37 °C for 4 hr and then at room temperature overnight. The deposit of each sample was seeded by Pasteur pipette into 3 tubes of Löwenstein–Jensen medium.

Results and discussion. Table I shows that several of the laboratory strains failed to grow, and two strains exhibited poor growth after chlorhexidine gluconate treatment. Organisms belonging to the same species, however, survived in clinical samples pre-treated with the agent. The difference may be due to the variable effect of the agent on different strains of the same species or to a difference in the number of bacteria in the model suspensions and in the

Table I

Survival of acid fast bacteria after exposure to chlorhexidine gluconate

Laboratory strains			Clinical samples in the years 1977–1978, organisms cultured
not recovered	poor growth	good growth	
M. smegmatis	*M. fortuitum*	*M. scofulaceum*	*M. smegmatis*
M. phlei	*N. asteroides*	*M. kansasii*	*M. phlei*
M. marinum		*M. chelonei*	*M. marinum*
M. gordonae		*M. xenopi*	*M. gordonae*
N. pellegrino		*M. avium*	*M. scofulaceum*
N. corallina			*M. kansasii*
N. rubra			*M. chelonei* complex
R. gordonae			*M. fortuitum*
			M. flavescens
			M. avium
			M. xenopi
			Nocardia sp.

clinical samples. The most probable explanation is, however, that slime and other substances present in clinical samples inhibited the effect of the agent.

The results have shown that pre-treatment of clinical samples with chlorhexidine gluconate allows to grow pathogenic and facultatively pathogenic mycobacteria and may, accordingly, be recommended as a routine diagnostic procedure. Nocardiae seem to be more sensitive and, accordingly, in suspected nocardiosis, in addition to the pre-treated material, an untreated sample should also be cultured.

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