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OPTIMUM APPLICATION OF LIQUID TWO-PHASE SYSTEMS FOR THE CONCENTRATION OF BACTERIOPHAGES

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(Received March 1, 1972)

Summary. Liquid two-phase systems suitable for the concentration of phages ΦX174, λ, and MS2 were applied and the measured phage distributions are presented. A simple semi-empirical method is described for the determination of phase diagrams necessary for the optimum application of the systems studied. This method has made it possible to describe the phase curves of the PEG6000-NaDS and PEG20 000-NaDS systems in the form of $y = \frac{c}{x + b} + c$ for an ionic environment like the synthetic medium M9 supplemented with 0.25 mole/litre NaCl.

The use of liquid two-phase systems for the concentration of macromolecules has become wide-spread since the middle of the present century [1]. This method which allows concentration with simultaneous purification without denaturation [2], is mostly applied as one of the numerous steps in the concentration process [3, 4]. Less steps are, however, needed if the phase diagrams of these systems are known since in this case the given particles can be concentrated in any volume.

Fig. 1 shows the phase diagrams of two-phase systems used by us. There are some common features of such diagrams: the ratio of the phase volumes for polymer concentrations belonging to point P is equal to the length ratio of sections CP and PD of the line running over point P (tie line). The points C and D, the points of intersection between the tie line and phase curve, indicate the polymer concentrations of the two phases in the solution of composition P. The slope of the lines is constant for a given system, independent of the choice of point P.

In the literature [5], the phase diagram is determined by measuring the polymer concentrations by chemical analysis in the different phases after the establishment of the two-phase condition using a series of different concentrations of solutions prepared from the same polymers. Our semi-empirical method to be presented in this paper facilitates the rapid determination of phase diagrams.

The phase diagrams have been determined for such kinds of systems which show distributions favourable for the concentration of the phages used, *viz.* the phage concentration was found extremely high in one of the phases or at the phase boundary.

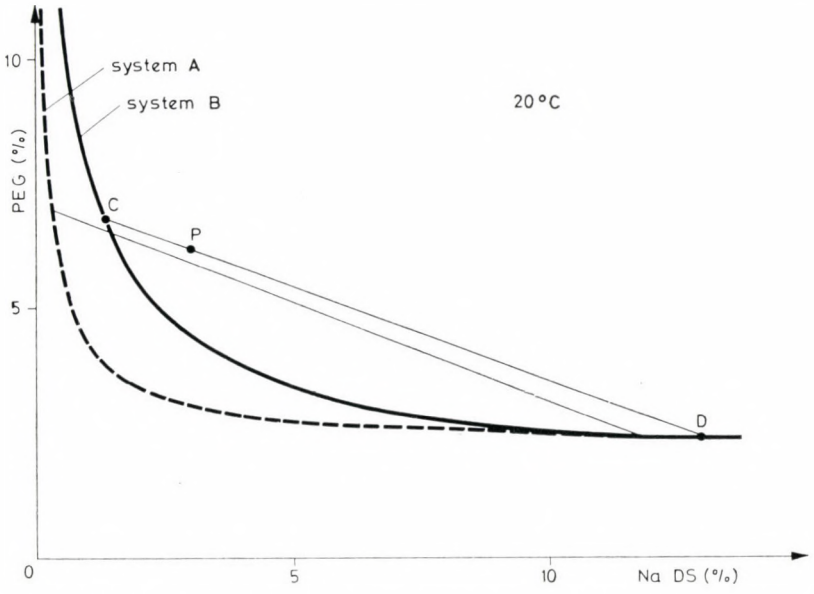

Fig. 1. Phase diagrams as determined by the semi-empirical method. System A: PEG20 000—NaDS, M9 + 0.25 mole/litre NaCl. System B: PEG6000—NaDS, M9 + 0.25 mole/litre NaCl

Materials and methods

Reagents. PEG (Reanal), of 6000 and 20 000 mol wt and NaDS (Pharmacia) of 500 000 mol wt were used.

For the determination of the phase diagrams, 30 solutions different in polymer concentration and stable in the two-phase stage, were prepared for both systems. Each solution was simultaneously made up to a final volume of 10 ml in graduate cylinders. After 24 hr at 20°C, the volume ratio of the phases developed was read.

The phage lysates to be concentrated were obtained from cultures in synthetic medium M9 [6]. Initial phage concentration was of the order of 10^{10}/ml.

Phage distribution was determined by a dripping method using systems with a total volume of 10 ml, where the volume of the lower phase was 1 ml. Besides the lower phase, the lower 1 ml of the upper phase was also dripped and the phage number was estimated in each of the drops (one drop was about 0.05 ml in size).

Results

Phase curves available in the literature [5] for systems similar to those used by us and also of certain other types (PEG20 000—D17, NaDS–methylcellulose) could be approached by the formula $y = \frac{a}{x+b} + c$. This finding yielded the basis of the semi-empirical determination of the phase diagrams developed by us, since the same approach has been accepted for the phase curves of the systems PEG20 000—NaDS and PEG6000—NaDS M9 + 0.25 mole/litre NaCl. In this case, for given values of a, b, c parameters and when the slope of the tie lines is equal to a certain m value, such a CP/PD value can be

read for any P points from the phase diagram drawn on the basis of the theoretical function curve, which is equal to the ratio of the volumes of phases developing under experimental conditions in a composition corresponding to point P.

It follows, that if the mathematical formula of the CP/PD quotient (referring to the given concentrations as point P and where points C and D represent the points of intersection between the $y = \frac{a}{x+b} + c$ function and the tie line running over point P with a slope equal to m) is put down for a solution of known composition (see Fig. 1, point P) and if this expression is compared to the value for the volume ratio measured at concentration P, then the deviation between the two values must be zero for certain values of the parameters a, b, c and m. Consequently, the desired values of a, b, c, and m are given by the minimum condition of an expression obtained if the differences between the measured volume ratios and the mathematical expressions of the appropriate CP/DP quotients found are composed for all of the prepared solutions of the system (30 solutions for both systems) and the squares of these expressions are summed for all solutions.

The approximate function formula of the phase curve was determined by a Hewlett—Packard computer from the above minimum condition. The following values were found: $a = 2.6$, $b = 0.22$, $c = 2.2$, $m = 0.4$ for the PEG20 000—NaDS system; and $a = 10$, $b = 0.53$, $c = 1.6$, $m = 0.38$ for the PEG6000—NaDS system. Fig. 1 shows these very phase diagrams. (The calculated and estimated values of the length ratio of the section of the tie line were in good agreement, showing a deviation of less than 10% for both approximations.)

Attempts were made to apply two-phase systems for the concentration of phages ΦX174, λ, and MS2. The situation for ΦX174 phages differed from that described in the literature [3] in that we used M9 phage-lysate as the solute of the system. The different ionic environment after the addition of 0.25 mole/litre NaCl was found to result in a favourable concentration similar to that described by SEDAT and SINSHEIMER [4].

Fig. 2 illustrates phage distribution as determined by the dripping method. Distribution of the MS2 phages may be seen in the same system. In the latter case, however, there is no increase in the concentration since no enrichment occurs at the phase boundary. Using polyethylene glycol of a sufficiently high molecular weight, a system can be obtained which allows the concentration of the MS2 phages. This is illustrated by Fig. 3, comparing the distribution pattern of MS2 obtained in 8% PEG20 000—1% NaDS system in M9 + 0.25 mole/litre NaCl to that found in PEG6000. It must be noted that the distribution of λ phages in the PEG6000—NaDS system is the same as that of the ΦX174 phages.

Figs 2 and 3 seem to indicate that phage concentration is not limited to the phase boundary or to its immediate vicinity, but covers a wider range where a high number of particles has been found. The phenomenon may be explained by an expansion of the highly viscous phase boundary during dripping, the effect of which prevails through several fractions. The actual number of particles developing at the phase boundary can be estimated by ordering

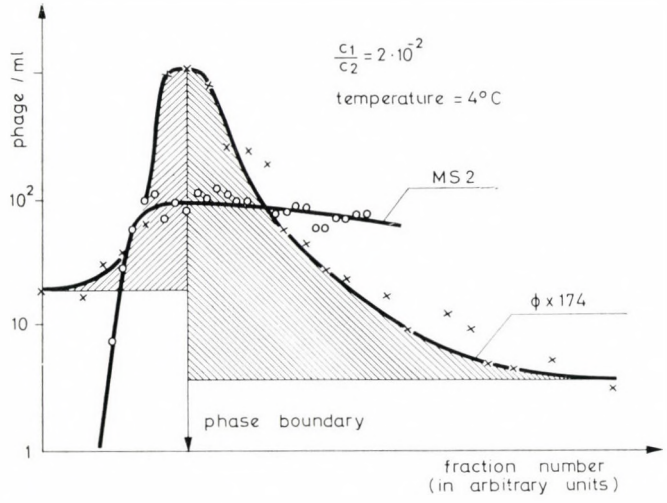

Fig. 2. Number of phages MS2 (○) and ΦX174 (×) in the drop fractions in the NaDS—PEG6000 system

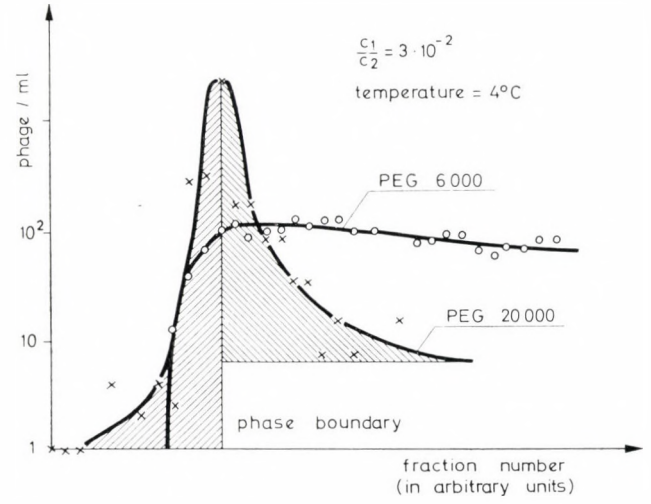

Fig. 3. Number of phages MS2 in the drop fractions using the NaDS—PEG6000 (○) and the NaDS—PEG20 000 (×) systems

the number of particles corresponding to the shaded areas of the figures to the boundary. In practice, the upper phase is usually decanted and the lower phase including the phase boundary is used for the estimation. In the above favourable cases, the ratio of the particle concentrations thus obtained is about 100.

Discussion

The results show that for the given bacteriophages two-phase systems have been found which in the case of an adequate composition result in a favourable distribution of the phages. The favourable formation of the phage concentrations in the phases does not yet mean that as compared to the initial concentration, the concentration is significant and the phage yield satisfactory. To this end, appropriate phase volumes must be chosen [4]. Our method for the rapid determination of the phase diagram allows a prediction of how the polymer concentration of a system with favourable distribution may be changed to reach the favourable distribution, and at the same time, the appropriate volume ratio. (This new optimum concentration is defined by that point of the tie line passing over the point corresponding to the composition giving favourable distribution which divides the tie line corresponding to the desired volume ratio.)

Since two-phase systems can be used not only for the purification and concentration of bacteriophages, but also for those of other macromolecules, viruses, cells, cell components, etc., and since our semi-empirical method for the determination of the phase diagram is also of general validity, it may help in the optimum utilization of the possibilities offered by the two-phase systems.

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COMPARATIVE ANALYSIS OF INTERLABORATORY VARIATION IN DRUG SENSITIVITY TEST FOR MYCOBACTERIUM TUBERCULOSIS

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(Received March 14, 1972)

Summary. Colony counting on drug-free media seeded with standardized inocula showed different standard deviation in three different laboratories using the proportion method of drug sensitivity test. The finding may explain the well-known fact that the results of sensitivity tests frequently differ in the different laboratories. Since in one laboratory the standard deviations showed no significant difference from the Poisson distribution, it seems probable that it can be reached not only for laboratory strains but also for freshly isolated cultures. Fitting the data obtained to the Poisson distribution allows the checking of the work of laboratories and the coordination of the methods.

In the proportion method for the determination of drug sensitivity of *Mycobacterium tuberculosis*, colony counting is influenced by the variance between samples [1]. To improve the results of drug sensitivity tests, Galvez-Brandon and Bartmann performed calculations using standard deviation data [2]. They assumed that the counts followed Poisson's law. The calculated and experimental results for strain $H_{37}Rv$ were in agreement.

For the evaluation of drug sensitivity tests carried out in our laboratory, an empirical method, taking into account the standard deviation has been applied [3]. In order to introduce the method based on calculations, the standard deviation for colony counts on drug-free control media was determined. These investigations showed that the standard deviation values observed did not fit Poisson's law [4]. The finding might partly be associated with the technique applied or, also with the fact that strains freshly isolated from patients were tested. One possibility of solving the problem was to compare the results of several laboratories obtained with standardized bacterium dilutions on drug-free media.

Materials and methods

Culture medium. Every laboratory was provided with Löwenstein—Jensen medium concentrate prepared in the central laboratory.

Statistical method. If the number of colonies grown on two drug-free media is denoted with X and Y, which are supposed to be independent Poissonian random variables of identical parameter, the distribution of the statistics

$$Z = \frac{(X-Y)^2}{X-Y}$$

could be approximated by a χ^2 distribution d. f. 1, serving to check whether the data are following Poisson's law. Applying this procedure the cultures were grouped according the total number of colonies ($:X - Y:$). The Z-values for each pair were summed within groups. In Table I f denotes the number of pairs and Q stands for the sum of Z statistics. The lower (0.05) and upper (0.95) significance limits for the Q values are also presented. The standard deviations were estimated by $\frac{Q}{f}$.

Table I

Determination of drug sensitivity tests of M. tuberculosis in different laboratories
Data of paired numbers of colonies grown on two parallel control media

Designation of laboratory	No. of colonies	f	Q	$\chi^2 0.05$	$\chi^2 0.95$	S^2
A 1969	31—40	50	97.75	34.8	67.5	1.95
	41—50	42	80.25	28.1	58.1	1.91
	51—60	32	41.32	20.1	46.2	1.29
	61—70	38	72.40	24.9	53.4	1.91
	71—80	38	145.26	24.9	53.4	3.82
	81—90	29	109.94	17.7	42.6	3.79
	91—100	34	88.45	21.7	48.6	2.60
A 1970	31—40	26	41.99	15.4	38.9	1.62
	41—50	41	75.39	27.3	56.9	1.84
	51—60	35	70.78	22.5	49.8	2.02
	61—70	34	80.13	21.7	48.6	2.36
	71—80	22	64.24	12.3	33.9	2.92
	81—90	38	127.40	24.9	53.4	3.35
	91—100	35	145.84	22.5	49.8	4.17
B	81—90	37	26.34	24.1	52.2	0.71
	91—100	40	13.31	26.5	55.8	0.33
	101—110	41	15.92	27.3	56.9	0.39
	111—120	25	15.00	14.6	37.7	0.60
C	0—10	31	40.68	19.3	45.0	1.31
	11—20	24	35.80	13.8	36.4	1,49
	21—30	16	20.76	7.9	26.3	1.30
	31—40	21	31.03	11.6	32.7	1.48

Origin of the material. Diagnostic Laboratory of the Korányi National Institute of Tuberculosis and Pulmonology; State Institute of Pulmonology, Mátraháza (Dr. L. CZABAFY) and County Pulmonological Institute, Zalaegerszeg (Dr. P. HEGYI). Calculations were accomplished with the Olivetti Programma 101 apparatus in the BCG Laboratory of the National Institute of Public Health, Budapest (A. NÉMETH, Dr. LUGOSI).

Results

The standard deviation values for Laboratory B were significantly lower than expected for Poissonian variables, presumably because of subjective errors committed in counting the colonies. In Laboratory A the standard deviation values were significantly higher than the Poissonian variables. In Laboratory C the two values showed no significant difference.

Discussion

The proportion method of investigating drug sensitivity depends not only on the concentration of the drug, but also on the ratio of resistant and sensitive bacteria. To achieve reliable ratios the variance for colony counts between samples should be considered [1—3]. This value differed between laboratories. This finding may explain the known fact that the results of drug sensitivity tests may differ according to laboratories. The present work has thrown some light on the reason for the difference and may serve to eliminate the errors. Thus, (a) the testing of Poissonian distribution may promote the checking of the work of laboratories; (b) the results may help in selecting improved methods.

Data for Laboratory C indicate that a Poissonian distribution may also be obtained in examining strains isolated from patients.

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HOMOLOGOUS INTERFERENCE OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS

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(Received August 22, 1972)

Summary. Monolayers of Ruzicska's III/1 monkey kidney cell line were infected with undiluted suspensions of the WE_3 strain of LCM virus. The virus yield was low and the harvested medium interfered with the growth of the homologous virus. Using the Armstrong E-350 strain of LCM virus in III/1 cultures or the WE_3 strain in primary monkey kidney cell cultures, the interfering activity of the harvested virus was less pronounced. Interferon had no role in this interference. Since the interfering substance is sensitive to UV irradiation and, according to other investigators, it is of particulate nature, the interference may be attributed to defective interfering (DI) particles. Interfering substance is produced simultaneously with the synthesis of the infective virus and its production continues after the maximum infectivity has been reached. The interference is very poor if the interfering substance is added to cell cultures 30 minutes or more after infection with the standard virus.

Among the interference phenomena described in the field of viruses, the homologous interference induced by incomplete, or defective interfering (DI), particles [1] is of special pathological significance.

The existence of DI particles has been demonstrated in the case of numerous viruses including influenza virus [2], vesicular stomatitis virus (VSV) [3], fowl plague virus [4], polyoma virus [5], Sendai virus [6] and poliovirus [7].

Based on their studies published in 1972, Welsh and Pfau [8] have suggested the role of DI particles in the homologous interference of the LCM virus. The present investigations have led us to the same conclusion.

Materials and methods

Viruses. The Armstrong E-350 strain [9] and the WE_3 line of the WE strain [10] were kindly supplied in mouse-brain suspensions by Professor F. Lehmann-Grube (Hamburg). In this laboratory, the strains were maintained in cell cultures without any further passage in mice.

Cell cultures. Primary rhesus monkey kidney (PMK) cell cultures and Ruzicska's [11] continuous rhesus monkey kidney cell line III/1 were employed as monolayers in tubes. As maintenance medium Parker's No. 199 enriched by bovine albumin was used. The virus-infected cell cultures were incubated at 37°C without rotation.

Infectivity titrations were carried out in III/1 cell cultures in tubes as described previously [12]. Besides the cytopathogenicity of the virus, the detection of viral antigens by immunofluorescence was used for the demonstration of viral multiplication.

Measurement of interfering capacity. III/1 cell cultures were inoculated with a twofold dilution series of virus suspensions. After an incubation for 2 hr at 37°C, each culture was superinfected with approximately 100 CPD_{50} of the strain WE_3. The interfering capacity was characterized by the highest dilution showing interference as indicated by a reduced cyto-

pathic effect. The decrease in the ratio of fluorescent cells was also taken into consideration in estimating the interfering capacity.

UV inactivation. Virus suspensions were irradiated with a germicide lamp from a distance of 20 cm for 5 or 10 min.

Immunofluorescence (IF) tests. For preparing conjugate, mice were hyperimmunized with LCM virus and the serum thus obtained was conjugated with fluorescein isothiocyanate (FITC, Serva, Component "A" rein) by incubation at room temperature and pH 9.5 at an FITC-globulin ratio of 1 : 150 for 1 hr. The conjugate was filtered on Sephadex G-50 column to remove the free FITC and subjected to chromatography on DEAE-cellulose column. The optimally labelled fraction was used for direct IF tests. Conjugate for indirect IF tests was prepared from goat anti-human-IgG serum. The cell preparations stained in the usual way were counterstained with 1 : 50,000-diluted Evans blue for 1 minute and covered with buffered glycerol. The preparations were examined in dark field with a monocular microscope in the blue light of the HBO-50 bulb.

The complement-fixation test was carried out using TAKÁTSY's microtitrator technique [13] and cold fixation overnight.

Results

(a) *Dose-dependence of the virus yield.* In previous studies [12] we have shown that the LCM virus multiplies and causes cytopathic changes in III/1 cell cultures. In the course of infectivity assays the cytopathic effect often failed to develop in cultures inoculated with large doses of the WE_3 strain. Alternatively, the early changes disappeared by the 5th day following inoculation. To clarify the backgrounds of this phenomenon, III/1 cell cultures were inoculated with undiluted or diluted inocula and both the soluble CF yield and the infectious virus yield were determined in the infected cultures. The CF antigen titres were found approximately identical while the infective titres were 100 to 1000 times lower in the cell cultures inoculated with the undiluted virus.

The undisturbed synthesis of the CF antigen concurrently with a strongly inhibited synthesis of the infectious virus suggests that noninfectious virus was produced in the cultures inoculated with undiluted virus.

To check this assumption, III/1 cell cultures were infected with various dilutions of the WE_3 strain and the infectious virus in the medium of several tube cultures was titrated 120 hours after inoculation. At the same time, cells from the infected cultures were examined by IF and the remaining cells were homogenized in order to prepare CF antigen.

Table I shows that the virus yield was considerably less in the cell cultures infected with undiluted virus. At the same time, the frequency of fluorescing cells was strikingly low. In experiments carried out with the E-350 strain, neither the infectivity nor the percentage of fluorescing cells was significantly diminished in the cultures inoculated with the undiluted virus, although a similar tendency was observable. It has therefore been concluded that the phenomenon under study is strain-dependent.

The titre of the CF antigen on the fifth day appeared not to depend on the size of the inoculum. On the basis of our earlier studies [12] this is because

Table I

Infectivity, fluorescing activity and CF of titre of III/1 cell cultures inoculated with different dilutions of LCM virus

Strain	Dilution of inoculum*	Infectivity* (CPD_{50}/0.1 ml)	Fluorescent cells, per cent	CF antigen** titre
LCM WE_3 2nd passage	0	3.75	1—2	8
	1	4.0	3—5	16
	2	5.0	20—25	16
	3	5.75	30—40	16
	4	6.25	40—50	16
LCM Armstrong, E-350 2nd passage	0	4.5	30—40	8
	1	5.0	50	8
	2	5.0	50	8
	3	5.5	75	8
	4	5.5	75	8
	5	4.0	20	4

* — log
** Reciprocals

the infectious virus in the cell culture reaches its peak between the 36th and 48th hr after inoculation with undiluted virus, whereas in cultures inoculated with diluted virus the peak is reached on the fifth day. The synthesis of infectious virus and that of the CF antigen runs parallel.

It was therefore assumed that in cultures inoculated with undiluted virus the production of infectious virions discontinues at the 48th hr in spite of the great number of uninfected cells; since the infected cells detach from the monolayer, the IF-positive cells diminish in number and in the fluid medium an interfering substance accumulates besides the infectious virions already present.

It might, however, be supposed that it is not the production of infectious virus that is inhibited by an accumulating interfering substance, but virions are inactivated by heat in the period from the 48th hr to the 5th day. We succeeded in excluding this explanation by the following experiment. Cell cultures were inoculated with undiluted WE_3 virus and, after the adsorption period, the cultures were washed at intervals to remove at least part of the hypothetic interfering substance. In such cultures the infectivity reached a titre at least 100 times as high as the titre of the cultures which had not been washed.

(b) *Development of the interfering activity in infected cultures.* III/1 cell monolayers were infected with undiluted suspensions of the WE_3 strain.

During incubation several tubes were taken out at intervals to determine the infectivity and the interfering activity of the culture medium, the frequency of the fluorescent cells and the CF antigen titres of cell homogenates (Fig. 1).

Infectivity of the medium reached its peak 48 hr after inoculation, then gradually declined; the frequency of fluorescent cells ran parallel with the infectivity. The CF antigen, too, reached its maximum at 48 hours and

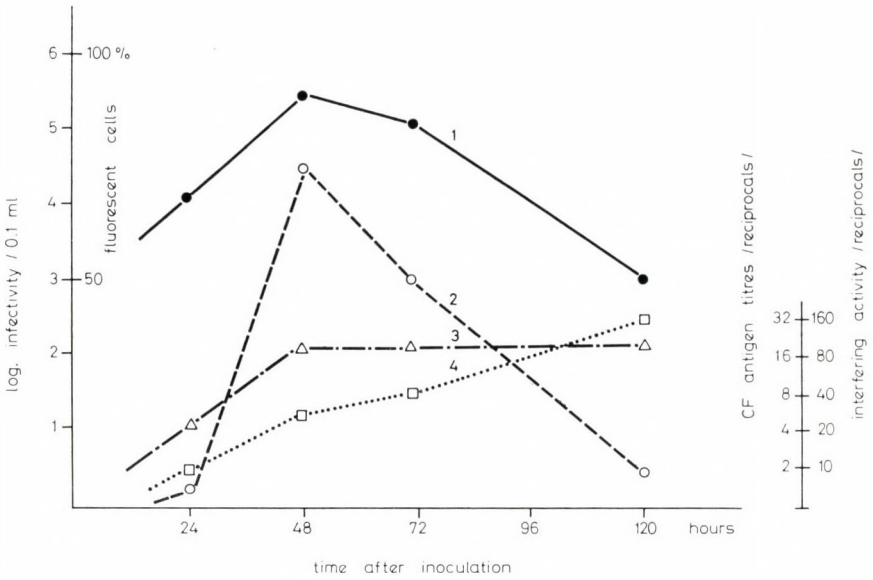

Fig. 1. Infectivity, percentage of fluorescent cells, CF antigen titre and interfering activity in III/1 cell cultures infected with undiluted WE_3 virus in function of time; 1 = infectivity; 2 = percentage of fluorescent cells; 3 = CF antigen; 4 = interfering activity

remained at that level until the end of the experiment at 120 hr. The interfering activity increased somewhat slower until the end of the experiment, the titres being 1 : 40 and 1 : 160 in the 48th and 120th hour, respectively. Accordingly, synthesis of the interfering substance continues even after infectious virions have stopped being produced.

(c) *Production of interfering substance in different host cells.* In the following experiments the WE_3 strain was subjected to serial passages with undiluted and 10^{-3}-diluted inocula in III/1 cell cultures and, in parallel, in PMK cell cultures. Infectivity and interfering activity of the medium were determined four days after inoculation (Table II).

The production of both factors was influenced by changing the host cell, and the interfering activity was more intensive in cultures inoculated with the undiluted virus than in those inoculated with the diluted virus. In the first "undiluted" passage in III/1 cell cultures, the infectivity was more than 100

Table II

Infectivity and interfering activity of WE_3 strain at different "diluted" and "undiluted" passage levels in different cell cultures

Passage No.	Dilution of inoculum	Passage in III/1 cell cultures		Passage in PMK cell cultures	
		Infectivity*	Interfering activity**	Infectivity*	Interfering activity**
1	undiluted	2.25	160	4.75	40
	10^{-3}	4.75	20	4.5	<10
2	undiluted	4.5	80	5.5	20
	10^{-3}	4.75	20	5.75	<10
3	undiluted	3.75	160	6.25	10
	10^{-3}	5.5	20	6.5	<10
4	undiluted	4.5	80	4.5	40
	10^{-3}	6.25	20	5.75	10

* — log
** Reciprocals

times lower in the culture fluid than in the respective "diluted" passage. The interfering activity was considerably higher in the III/1 line than in the PMK cultures. The fluctuation of the infective titre of the WE_3 strain as passaged in III/1 cultures is of special interest. A similar fluctuation has been shown in cell cultures persistently infected by the vesicular stomatitis virus [1]. It can be concluded that, similarly to other viruses, the production of DI particles of the LCM virus depends on the host cell.

(d) *Production of LCM virus in vivo.* DI particles have never been demonstrated in the course of natural virus infections of animals. The following experiment shows that the DI particles and the interference phenomenon attributable to them cannot be considered a mere artefact. Adult mice were inoculated intraperitoneally with 20 million cells of the S-37 mouse sarcoma cell strain [12]. Twenty-four hours later half of the mice were inoculated intraperitoneally with an undiluted suspension of the WE_3 virus grown in III/1 cell cultures, while the other half with diluted inocula (approximately 10 CPD_{50}/mouse) of the same virus suspension. On the 2nd, 4th and 6th day after infection three mice each were killed and their ascites fluids were pooled and centrifuged. In the supernatants, infectivity and interfering activity were determined, the cells were counted and smears were prepared for IF test. Results are shown in Table III.

We have shown [12] that the LCM virus multiplies in S-37-sarcoma-bearing mice not only in the normal mouse tissues but also in the sarcoma cells. In the present experiments, in the mice that had been inoculated with

the larger dose of the virus, the sarcoma cells ceased multiplying after the LCM infection, half of them contained fluorescent antigen and the ascites fluid elicited an unusually high infectivity and a high interfering activity. In the mice which had been inoculated with the smaller dose of virus, the virus grew

Table III

Infectivity, interfering activity and percentage of fluorescent cells in ascites fluid of mice inoculated with S-37 mouse sarcoma cells infected with WE_3 virus

Time of harvesting ascites fluid*	Infection with 10^4 CPD_{50}/mouse				Infection with 10 CPD_{50}/mouse			
	No. of ascites cells (million)	log infectivity per 0.1 ml	Interfering activity**	Fluorescent cells, per cent	No. of ascites cells (million)	log infectivity per 0.1 ml	Interfering activity**	Fluorescent cells, per cent
2	80	4.75	100	2—5	80	3.50	<10	0
4	120	>8.50	1000	50	200	4.50	<10	0.5—1
6	100	6.75	1000	50	800	4.25	<10	0.01

* Days after virus infection
** Reciprocal dilutions
Sarcoma cell inoculum: 20×10^6 cells intraperitoneally, virus inoculum from the 16th passage on III/1 cell cultures, administered intraperitoneally 24 hours after inoculation with sarcoma cells

mainly in the mouse tissues, the interfering activity of the ascites fluid was negligible and the sarcoma cells continued to multiply. It seems reasonable to suppose that the interfering substance, presumably consisting of DI particles, was produced by the S-37 sarcoma cells.

(e) *Identity of the interfering substance with DI particles.* The identity of the interfering substance with DI particles is supported by indirect evidence. The above-described experiments have suggested that the substance responsible for the interference phenomenon is not identical with the virion or with the soluble CF antigen.

(1) *The interfering substance is not interferon.* III/1 cell cultures were inoculated with LCM virus suspensions of high interfering activity and superinfected with 50 CPD_{50} of VSV 24 hours later. No inhibition occurred, whereas the interferonogenic agent poly I : C considerably inhibited the growth of the same VSV in III/1 cell cultures. Welsh and Pfau [8] have shown that the substance responsible for the homologous interference of the LCM virus must be of approximately the same size as the virion itself.

(2) *The interfering substance is sensitive to UV irradiation.* Assuming the particulate character of the interfering substance, we cannot exclude *a priori* that the interference is of competitive nature being caused by adsorption of cell debris on the surface of susceptible cells. The fact that the substance is sensitive to UV irradiation (Table IV) excludes this objection.

Table IV

UV-sensitivity of the infectivity and interfering activity of WE_3 LCM virus

Virus	Infectivity*	Interfering activity**
Untreated	4.5	160
Irradiated for 5 min	<0.5	<10
Irradiated for 10 min	<0.5	<10

* — log
** Reciprocals

A decrease in the infective titre of the virus was accompanied by a decrease in its interfering activity. The UV sensitivity of the interfering substance suggests that the viral nucleic acid plays a role in the interference and thus offers a further indirect evidence for the role of DI particles. Nevertheless, the undiluted virus suspension retained some of the interfering activity even after 10 minute UV irradiation. This might be attributed to reactivation.

(f) *Time dependence of the interference.* The following experiment was aimed at revealing the mechanism of action of the homologous interference. The time of the administration of both of the interfering and superinfecting virus was varied. III/1 cell cultures were infected with a twofold dilution series of the interfering virus and superinfected with 100 CPD_{50} of standard virus. The time intervals were —24, —1, then 0, 1/2, 1, 2 and 8 hr as related to the time of the superinfection. The results are shown in Table V.

The interfering effect was the most intensive when the interfering virus have been administered before the superinfection irrespective of whether the

Table V

Time-dependence of the homologous interference of LCM virus in III/l cell cultures

Time of the inoculation of interfering virus before (—) or after (+) superinfection, hr	Interfering activity*
—24	160
—1	160
0	80
+1/2	20
+1	20
+2	10
+8	1

* Reciprocals of the highest virus dilution causing interference (inhibition of cytopathic effect)

time interval was 24 hours or 1 hour. This suggests that the interfering substance — probably DI particles — must reach some intracellular site(s) or affect some metabolic process early enough to exert its effect. If the interfering virus was administered simultaneously with, or after, the standard virus, the interference was the weaker the longer was the interval between the two inoculations. A very slight interference took place even if the interfering virus was added 8 hr after inoculation with the standard virus.

Discussion

The *in vitro* homologous interference of the LCM virus appears to be analogous to the interference between DI particles and infective virus demonstrated for other viruses. According to the hypothesis of HUANG and BALTIMORE [1] DI particles may arise as natural mutants of viruses. If such particles have appeared they begin to multiply at the expense of the complete virus; at last, they will prevail and a well-defined interference ensues. Thus, the process of infection discontinues. This mechanism besides specific immunity, may play a role in the recovery from viral diseases.

The role of DI particles may be of particular importance in persisting virus infections. It is well-known that mice infected with LCM virus in their intrauterine life or one day after birth, unlike those infected in adult age, do not die, but they become tolerant to the virus and the virus may persist in them lifelong [15]. Interferon plays no role in this persistence [16, 17], and the LCM virus does not induce interferon production in cell cultures. DI particles may be assumed to have an important role in persistent LCM virus infection.

Persistent LCM virus infection has been brought about in L-cells as well [18]. Although virulent virus was not produced after several passages, the inoculated cell cultures continued producing an agent of large molecular weight which interfered with the multiplication of the standard LCM virus.

We have observed that LCM virus strains may differ in their interfering, *i.e.* DI-particle-producing, capacity. Furthermore, the quality of the cell culture plays a role in the development of virus persistence: the homologous interference with the WE_3 strain was always less intensive in PMK cell cultures than in III/1 cells. The greatest interfering activity and, consequently, the lowest infective titres on the 5th day were obtained in cell cultures inoculated with large doses of virus. As to the appearance of the interfering activity and its chronological relationship to virus multiplication, our results are in accordance with those published by WELSH and PFAU [8], though we counted the IF-positive cells instead of the infection centres: after the peak of the infectivity curve the interfering activity increased further, while the IF-positive cells were already declining in number.

The IF-positive cells corresponded in number to the cells infected by the standard virus alone or by both the standard virus and the DI particles; the cells which have taken up only DI particles do not fluoresce because of the lack of virus multiplication in them. The antibodies taking part in the IF reaction were found to be different from the CF antibodies [14]. Examining patients' sera, we have demonstrated that the fluorescent antibodies appeared earlier than the CF antibodies.

We obtained virus suspensions of high interfering activity *in vivo* when mice were infected artificially with both mouse sarcoma cells and LCM virus. Though this system is different from the naturally infected animal, the results support the view that a viral product containing DI particles can be formed *in vivo* as well.

There are several indirect proofs of the identity of the interfering agent with DI particles. Our experiments support the earlier conclusion that interferon plays no role in this interference. It was found to be a particulate substance which sediments together with the infectious virus; its UV sensitivity is suggestive of a nucleic acid component.

As regards the mechanism of the homologous interference of LCM virus, our experiments concerning the time-dependence of the interference offer some information. We suppose that the interfering agent must reach some intracellular site early enough to interfere with the productive cycle. If it is administered after the standard virus, its effect is weak, suggesting that the blocking of virus multiplication takes place soon after the attachment and the penetration of the virion.

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ABSTRACTS OF PAPERS

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Bacteriology

THE ROLE OF L-SERINE IN THE REGULATION OF RNA SYNTHESIS OF ESCHERICHIA COLI

Cs. Kari, Gy. Bajszár, Enikő Kuthy and L. Alföldi

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It is almost generally accepted that each of the biologically important amino acids plays an equal role in the regulation of RNA synthesis in *Escherichia coli*. The present experiments are suggestive of the outstanding role of L-serine in this regulation mechanism. During serine starvation the relaxed mutants synthesized approximately as little RNA as the stringent mutants, even with adenine-less mutant in the presence of the five purine-pyrimidine bases and all the other amino acids. The cause of the phenomenon has not yet been clarified.

ATTEMPTS AT PRODUCING THERMOSENSITIVE ESCHERICHIA COLI MUTANTS IN THE REGULATION OF RNA SYNTHESIS

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RNA synthesis of *Escherichia coli* is regulated by amino acids in an unknown way. It has been assumed that the mechanism of regulation could be clarified by experiments with thermosensitive mutants in the regulation of RNA synthesis. A method has been evolved for a selective enrichment of such mutants. The principle of the method is as follows. During amino acid starvation relaxed mutants can selectively be killed with streptomycin and/or actinomycin D, whereas stringent cells are selectively sensitive to penicillin and/or thymine-less death. Combining these treatments with cells of suitable heat-sensitivity it seems possible to obtain the desired mutants.

STUDY OF A SPECIALLY TRANSDUCING RHIZOBIUM PHAGE

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The 16—3 temperate phage specially transduces the cysteine gene of *Rhizobium*. The gene locus of bacterial DNA incorporation into the transducing 16—3 phage was investigated. It has been examined which of the genes of cysteine biosynthesis are contained in the incorporated bacterial DNA. The genes of the transducing phage were demonstrated by complementing the defective prophage, being present in the cis^+ transductants, with heat sensitive mutants (located at 7 different loci of the phage chromosome which characterized it in its whole length). The experiments revealed that the immunity region and the late genes are missing from the transducing phage. Among four bacterial mutants defective in different genes of cysteine biosynthesis, the 16—3 phage could transduce the biosynthesis of cysteine into two bacterial mutants.

GENETIC ANALYSIS OF MUTATIONS INFLUENCING THE REGULATOR GENE FUNCTION OF TEMPERATE BACTERIOPHAGES

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From a heat-inducible mutant of *Rhizobium meliloti* 16—3 bacteriophage double mutants have been isolated which could be induced only at a higher temperature than the original one. The aim of the investigations was to show whether the thermoresistance of the repressor of the double mutants was elevated or the repressor molecules increased in number (super-repressive mutant). The double mutants are characterized by (i) a sensitivity to superinfection of bacteria lysogenized by the mutants, using a phage defective in the operator gene; (ii) the quantity of heat necessary for induction; (iii) determination of the mutation loci by two and three point crossings. It was rendered probable by mapping that there are mutants producing a protein of higher heat resistance and others overproducing repressor molecules (mutation of the promoter gene). The system allows the genetic examination of the promoter locus.

LYSOGENIC CONVERSION OF BACILLUS CEREUS: ISOLATION OF A PHAGE CAUSING PHOSPHOLIPASE PRODUCTION

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Some 15 years ago, the authors described megacin, a bacteriocin of *Bacillus megaterium*. Subsequently, Japanese authors have identified the megacin produced by the strain *B. megaterium* No. 216 as phospholipase A. Recently, the authors have noticed that some strains of *B. cereus* are lysed in the presence of mitomycin and a factor identical in its biological activity with megacin appears in the lysate; the factor proved to be phospholipase A. From one of these strains megacin-minus *(cin⁻)* cells segregated in the presence of acridine orange at a frequency of 5%. From the wild strain, which was carrying several prophages, an unknown phage has been isolated which did not lyse the usual indicators and formed plaques on cured, *i. e. cin⁻*, strains exclusively. The plaques were extraordinarily turbid in appearance. If exponentially growing liquid cultures of the *cin⁻* strain were inoculated with this phage, and at the same time mitomycin (0.5 μg/ml) was added to the cultures, they were lysed after a residual growth, producing a lysate of high megacin content. From the same phage a mutant forming clear plaques could be isolated which lysed the inoculated cells, producing complete phage particles, but no megacin. It is supposed that in the cells carrying the temperate phage, virion reproduction is prevented by a repressor and the cells produce phospholipase A instead of virions. If, however, the repressor undergoes mutation, the normal structural elements of the phage will be produced.

MECHANISM OF HELPER PHAGE TRANSFECTION

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Our helper phage transfection system elaborated for the bacteriophages of *Rhizobium meliloti* was used for the investigation of the role of the helper phage's protein and DNA and for the examination of the process of helper phage infection. According to our investigations, the process of helper phage transfection is as follows. (i) The helper phage absorbed to the bacterium induces DNA uptake in it. (ii) The DNA of the helper phage inactivates the restriction mechanism of the bacterium against the transfecting DNA. (iii) Transfectants occur in the cell; this can be the result of several processes: — "marker rescue", the helper DNA will be repaired by the recombination of the transfection DNA — the transfection DNA becomes reactivated by the

genes of the helper phage — the transfection DNA becomes reactivated without the cooperation of the helper phage's DNA. The transfection system can be applied for the examination of the functional and physical order of bacteriophage genes.

EXPRESSION OF EPISOMAL CHLORAMPHENICOL RESISTANCE IN ESCHERICHIA COLI AND SALMONELLA TYPHI-MURIUM STRAINS

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Chloramphenicol acetyltransferase (CAT) production has been studied in both cyclic 3,5'-adenosine monophosphate (cAMP) and cAMP receptor protein (CARP) deficient mutants. Addition of cAMP increased both CAT production and resistance to chloramphenicol in the cAMP deficient mutants. Simultaneous addition of cAMP and chloramphenicol caused a further increase in CAT production. Chloramphenicol alone caused an increase in the CAT production of the parent strains and their cAMP and CARP deficient mutants

THE ROLE OF CYCLIC 3,5'-ADENOSINE MONOPHOSPHATE IN THE COLICIN PRODUCTION OF ESCHERICHIA COLI

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Colicin production was measured in cyclic 3,5'-adenosine monophosphate (cAMP) deficient mutant strain GP-1 grown on casamino acids–yeast extract medium. Addition of cAMP increased colicin production. Grown on glucose as carbon source, colicin production was higher in the presence of cAMP.

COMPARATIVE STUDIES ON THE ULTRASTRUCTURE OF METHICILLIN-SENSITIVE AND METHICILLIN-RESISTANT MUTANTS OF STAPHYLOCOCCUS AUREUS

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The ultrastructure of cells of a methicillin-sensitive and a methicillin-resistant mutant, both derived from the naturally occurring methicillin-resistant *Staphylococcus aureus* strain 5814/1966 of mixed population, was

comparatively examined by electron microscopy. Measurements of cells were quantitated and analysed statistically. The cells of the same stage of both mutants were uniform in shape and size, showing coccal or elliptical forms. All the corresponding measurements of the two mutants were very close to each other except for the side wall appearing to be significantly thicker in the methicillin-resistant mutant. The thickening was in most part due to the widening of the middle dense layer of the wall. The ratio of side wall volume to protoplast volume was also significantly higher for the methicillin-resistant mutant. Arrangement of the nuclear material was different in the two mutants. There was no qualitative difference in cross wall formation nor in cell separation between the two mutants.

COMPARATIVE STUDIES ON THE EFFECTS OF METHICILLIN ON THE ULTRASTRUCTURE OF METHICILLIN-SENSITIVE AND RESISTANT MUTANTS OF STAPHYLOCOCCUS AUREUS

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The cells of the methicillin-sensitive mutant 5814S, cultured in the presence of methicillin (0.5 μg/ml) varied much in size and shape, and were significantly enlarged. Cross walls were widened and structureless. In a number of cells cross wall formation was uncontrolled and cell division was disorganized and imperfect, resulting in giant cells of various shapes. The cells of the methicillin-resistant mutant 5814R cultured in the presence of 800 μg methicillin per ml did not significantly differ in shape and size from the controls. Only the mean side-wall thickness, side-wall volume and the ratio side wall volume to protoplast volume increased significantly. Cross wall formation and cell separation were controlled. Division of DNA seemed to be unaffected by methicillin in both mutants.

THE PHAGE RESTRICTION EFFECT OF THE R FACTORS IN SHIGELLA FLEXNERI STRAINS

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A study was made on the resistance to antibiotics of 460 *Shigella flexneri* strains originating from patients during the year 1972. Of the strains 27.6% proved resistant to 1—3 antibiotics; 83 out of 106 resistant strains (78.3%)

carried R factor. The type of the R factor was *fi*$^+$ in 14 cases and *fi*$^-$ in 69 cases. The R factor was transferred to the same *Escherichia coli* strain (HfrH) sensitive to antibiotics and to sensitive *S. flexneri* strains. The phage sensitivity of *E. coli* strains was examined with 28 phages used for phage typing, that of *S. flexneri* strains with 19 *S. flexneri* type phages. Among the *E. coli* phages, the 1, 2, and 6 phages, among the *S. flexneri* phages, the 1, 2, 3, 6, 7 and 9 phages were restricted. There was a correlation between restriction and the serological properties of phages. Restriction occurred both after *fi*$^+$ and *fi*$^-$ R factor transfer. Strains different in resistance to antibiotics and in phage sensitivity were isolated from the same patient on two occasions. Transfer of the R factor from the strain resistant to antibiotics into the sensitive one caused the phage type to change. The *in vitro* transformed strain had the same phage sensitivity and was of the same resistance to antibiotics as the resistant strain occurring *in vivo*.

ANTIBACTERIAL EFFECT OF OLEFICIN ON STAPHYLOCOCCUS AUREUS STRAINS

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The mechanism of action of oleficin, a new polyene-type antibiotic was investigated. It was effective against Gram-positive bacteria and had a weak cancerostatic effect. Growth of *Staphylococcus aureus* Duncan bacteria in the log phase was stopped by oleficin at 0.7 μg/ml concentration. [^{3}H] Thymidine, [^{14}C] uracil and [^{14}C] leucine incorporation into DNA, RNA and protein macromolecules, respectively, was measured. Inhibition occurred simultaneously and was of the same degree when the above concentration was used. In the course of investigation into cell wall synthesis by the method of Park and Hancock it was found that [^{14}C] glycine incorporation into the murein structure was stopped immediately while incorporation into the cytoplasmic proteins continued. The antibacterial effect of oleficin is based probably on its inhibitory effect on the cell wall synthesis of bacteria.

QUANTITATIVE ASPECTS OF PROTOPLAST FORMATION AND REGENERATION IN YEASTS

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The authors examined the conditions of protoplast formation and regeneration in 40 yeast strains belonging to the genera *Endomyces*, *Schizosaccharomyces*, *Debaryomyces*, *Endomycopsis*, *Pichia*, *Hansenula*, *Dekkeromyces*,

Geotrichum, *Candida*, *Torulopsis*, *Kloeckera*, *Cryptococcus* and *Rhodotorula*. Almost 100% of the cells were converted into protoplasts, in the majority of strains within 3 hr, if log-phase cells were treated with an enzyme mixture from *Helix pomatia* in 0.6 M KCl solution at pH 7.0 after pretreatment with 1% β-mercaptoethanol. Regeneration to normal cells and colonies can be accomplished with high efficiency on isoosmotic agar media after covering the protoplasts plated on the surface with a thin agar-layer by spraying. Developing colonies could be transferred by replica plating. With the above technique, fusion of protoplasts and regeneration of the fused cells could be achieved. This is the first biochemically proven case of protoplast fusion with fungal cells.

LETHAL AND MUTAGENIC PHOTOSYNTHESIZING EFFECT OF 8-METHOXY-PSORALEN AND UV_I LIGHT (365 nm) ON ESCHERICHIA COLI DIFFERENT IN DNA REPAIRING CAPACITY

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Furocoumarin of plant origin assumes a covalent bond with the pyrimidine bases of DNA under the effect of fluorescent light. The cells can be protected from most of the lethal and mutagenic consequences of these photoadducts by their repairing enzyme system. There was a difference of almost one order of magnitude in survival and in mutation frequency of *Escherichia coli* B/r WP2 *trp*$^-$ *hcr*$^+$ strains having excision capacity and *hcr*$^-$ *E. coli* strains devoid of this capacity following UV irradiation of 365 nm wavelength in the presence of 8-MOP (50 μg/ml). The biological effect of UV light of 254 nm wavelength was diminished by 8-MOP (100 μg/ml). The protective effect was of one order of magnitude in the case of lethality, of mutation of the *hcr*$^+$ strain, and of the mutation frequency of the *hcr*$^-$ strain, and it was of two orders of magnitude in the case of lethality of the *hcr*$^-$ strain.

COMPARATIVE IMMUNODIFFUSION ANALYSIS OF THE ANTIGENIC STRUCTURE OF MYCOBACTERIUM AVIUM AND MYCOBACTERIUM INTRACELLULARE STRAINS ISOLATED FROM DIFFERENT SOURCES

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The antigenic structure of *Mycobacterium avium* and *M. intracellulare* strains isolated from fowls and Asian monkeys was studied by immunodiffusion and immunoabsorption techniques. Strain differences were demonstrated, on

the basis of which certain strains within both species are well distinguishable. On comparison with other mycobacterial species, it was found that *M. avium* and *M. intracellulare* show the closest antigenic relationship to *M. simiae*, a species isolated from Asian monkeys and described by the authors.

RELATIONSHIP BETWEEN THE SPUTUM-WASHING PROCEDURE AND THE CYTOLOGICAL FINDING

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Two purulent expectorates were taken from each of 23 patients at an interval of 1 to 2 hours. Each sputum was divided into three parts and these were washed separately. The flocculi thus obtained were examined by bacteriological and cytological methods. The three parallel cultures yielded in each case the same microorganisms, whereas the parallel cytological results were variable for the majority of the sputa. Mouth toilet prior to expectoration did not influence the results.

INTRODUCTION OF STANDARD TECHNIQUES INTO THE EPIDEMIOLOGICAL BACTERIOLOGY LABORATORIES OF THE PUBLIC HEALTH SERVICE

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The advantage of standard techniques in medical diagnostic laboratories and in particular in epidemiological bacteriology is discussed. The efforts of the Department of Bacteriology of the National Institute of Public Health were actively supported by the Public Health Laboratories and received much attention from the Ministry of Health. As a result, a Guide to Bacteriological Methodology has been published, which prescribes the use of standard methods. The planned additions aiming at a further development of standardization are outlined. These include the use of dehydrate media, the introduction of industrially prepared biologicals (conjugated antiglobulins) meeting high quality demands. The Guide should be made to cover the activity of the laboratories of hospitals and out-patient clinics.

QUALITY CONTROL TESTS IN THE PUBLIC HEALTH BACTERIOLOGY LABORATORY NETWORK

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In 25 laboratories of the Public Health Service 34 different test materials were examined in the years 1964—1972: 10 different clinical materials and 3 faecal samples for cultural examination, 8 sera for antibody titre determination, 7 strains for identification, and 6 strains for antibiotic sensitivity testing. After the compulsory introduction of standard methods in 1971, these tests have revealed that the results of more special examinations (identification of rare pathogens, anaerobic cultivation) do not reach the medium level. In the year 1971, identification of pathogens yielded excellent results in 52.3%, good results in 23.3%; determination of the sensitivity to antibiotics was excellent in 22.3% and good in 26.3%. The time taken by the examinations yielded fairly good results in that 72.9% were excellent and 15.6% good.

PATHOMECHANISM OF INFECTIONS CAUSED BY FACULTATIVE PATHOGENS

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In recent years, microorganisms having been considered facultative Pathogens have had an increasing aetiological importance both in Hungary and abroad. This was especially so with respiratory diseases such as atypical mycobacteria, pulmonal mycoses and mycoplasmoses, which occurred in increasing numbers. Microorganisms formerly held to be saprophytes were isolated from more and more cases of chronic bronchitis and other respiratory diseases. The aetiological role of these microorganisms has often been confirmed by positive haemocultures. The increasing number of such cases can be attributed to (i) unnecessary antibiotic treatments; (ii) fluctuation of the blood iron level; (iii) fluctuation of the gamma globulin level, etc. The importance of a close collaboration between clinicians, microbiologists and of the use of modern microbiological procedures is stressed.

SOME OBSERVATIONS WITH YERSINIA ENTEROCOLITICA STRAINS AND ON THEIR ROLE IN HUMAN INFECTIONS

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In Hungary the first biochemical confirmation and serological identification of a *Yersinia enterocolitica* strain occurred in 1969. From that time till September 1, 1972, 275 strains were isolated and identified from 187 persons. Three strains were serotype O9 (Winblad's scheme) and biotype 3 (Niléhn's scheme); 175 were of serotype O3 and biotype 4. The biotype of 9 strains belonging to serotype O3 needs repeated determination. Heat resistance studies showed that *Y. enterocolitica* in aqueous medium withstood treatment at 50°C for 7 hours, but was killed at 60°C in one hour and at 70°C in 1 to 2 minutes. Formaldehyde exerted bacteriostasis at a concentration of 1 in 12 000; phenol at 1 in 800; Merfen at 1 in 400 000; sublimate at 1 in 300 000; cetylpyridinium bromide at 1 in 140 000. For a complete kill of *Y. enterocolitica* with 3% formalin, 7.5 minutes; with 5% phenol, 0.3 minutes; with 0.1%cetylpyridinium bromide 2 minutes; and with 0.1% chloramine B (in acid solution), 4 minutes were necessary. All the strains were sensitive to penicillin, oxacillin, novobiocin, ampicillin, spiramycin, oleandomycin, lincomycin; 62% to erythromycin. Sensitivity to chloramphenicol, neomycin, polymyxin-B, streptomycin, tetracyclines, gentamicin, colistin and kanamycin varied between 85 and 100%. Of the patients, 110 had diarrhoea as the main symptom, which was associated with fever, abdominal pain and occasionally with gastric signs. Only abdominal pain was present in 3 cases. Four patients had gastritis without diarrhoea. Symptoms suggestive of appendicitis were detected in 5 cases. One patient had erythema nodosum and arthralgia was reported in one case. In some patients, enteritis was preceded by respiratory tract symptoms and influenza-like signs. Other symptoms were recorded in 4 cases. In 17 cases no data on the clinical features were available and 42 persons excreting *Y. enterocolitica* were asymptomatic.

EXPERIMENTAL LISTERIOSIS IN ORALLY INFECTED NEWBORN ANIMALS

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The foetopathogenic effect of *Listeria monocytogenes* is shown by the fact that 1—2-day-old newborn pigs, rabbits, rats and mice can successfully be infected with *L. monocytogenes* by the oral route. These animals are then

retarded in growth and some of them — depending on the infective dose — die with septicaemia in 2—5 days. One-week-old suckling animals overcome the oral infection, but *L. monocytogenes* can be demonstrated from their faeces for a few weeks. Superinfection of these animals revealed only a small degree of immunity against *L. monocytogenes*.

FLAGELLATION OF HAEMOLYSING AND NON-HAEMOLYSING LISTERIA MONOCYTOGENES STRAINS

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In the course of screening tests non-haemolysing *Listeria monocytogenes* strains were isolated from the faeces of slaughter-house workers, from the upper respiratory tract of healthy pigs and from emergency-slaughtered animals. These strains were apathogenic to experimental animals. Flagellation was examined in these and in haemolysing pathogenic strains, in a total of 45. The investigation was carried out in semisolid stab cultures at 20, 37, and 40°C in parallel. There was a close correlation between flagellation and haemolysing capacity of the strains. At 20°C every strain produced flagella, at 37°C only the non-haemolysing ones. At 40°C all strains failed to produce flagella. This phenomenon was most expressed after a 24 hr incubation period.

INVESTIGATION OF BACTEROIDES FRAGILIS STRAINS. ISOLATION AND IDENTIFICATION OF BACTEROIDES IN CLINICAL SAMPLES

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Detailed studies have been made of the cultural, morphological and biochemical characteristics of 19 *Bacteroides fragilis* strains of human origin. In the presence of haemin all strains grew well in fluid media and a slight catalase activity was observed. Bile was stimulating growth in 20%. Among the other properties examined, the following were found of value in differentiation. *B. fragilis* is a strict anaerobic Gram-negative rod with more or less rounded ends, showing moderate pleomorphism. The organism is not motile, haemolysis was not observed. A small amount of gas and strong acid (pH 5.0—5.4) is produced at the fermentation of glucose. Clot reaction is given on litmus milk in 4 to 5 days. Hydrogen sulphide is formed. *B. fragilis* does not liquefy gelatin, does not reduce nitrates and is non-proteolytic. All the strains investigated were indole-negative. Glucose, maltose, lactose, sucrose, starch

and dextrin were fermented, but not arabinose, rhamnose, ribose, salicin, trehalose and glycerol. The chief products of fermentation were acetic and lactic acids, the amount of propionic, valeric and succinic acids was variable. From human clinical material 31 bacteroides strains were isolated of which 18 corresponded to the above definition. Fourteen strains originated from wound infection and 11 from gynaecological samples. All the strains were resistant to penicillin, 82% of the isolates were sensitive to chloramphenicol, 79% to erythromycin and 72% to tetracyclines. The majority (89%) of the strains was polymyxin-B resistant.

THE IMPORTANCE OF GRAM NEGATIVE BACTERIA IN THE ORIGIN OF OTITIS MEDIA OF PREMATURE INFANTS AND BABIES

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In a total of 40 premature infants and 160 babies suffering from otitis media the tympanic exudate was examined. The external auditory meatus was cleaned, disinfected, anaesthetized and the tympanic membrane was punctured with a short bevelled cannula. This was connected with a 2 ml syringe and a suction apparatus. The exudate obtained was immediately injected into Holman's culture medium and incubated at 37°C. Transfers were made after 24 hr and, if this sample was sterile, another subculture was made after 48 hr. The procedure seemed appropriate for culturing the pathogenic agent of otitis media without iatrogenic contamination. Results were reliable even if the patient was treated with antibiotics or the exudate contained very few bacteria. Gram-negative bacteria could be isolated from 80% of the prematures and 30% of the infants.

IMMUNOFLUORESCENT TECHNIQUE IN THE DIAGNOSIS OF ESCHERICHIA COLI SEROTYPES ASSOCIATED WITH INFANTILE DIARRHOEA

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From antisera prepared against alcoholized acetone-dry antigen of *Escherichia coli* O26, O55, O86 and O111, conjugates for direct immunofluorescent staining were produced according to Kawamura and Cerderberg. Chemical characteristics (protein concentration, fluorochrome content, fluorochrome–protein ratio, staining activity of the conjugate) and immunological

properties (working dilution, cross reactions, one-step inhibition test) were determined. Faecal samples were mixed with cultures of *E. coli* strains of the above serotypes and of *Salmonella* and *Shigella* strains. Using GERDERBERG's indirect immunofluorescent technique, smears of the samples were stained with polyvalent conjugate prepared from the 4 labelled antisera. For the observation an Olympus-VANOX type fluorescence microscope was used. From each sample parallel cultivation was performed. Both cultivation and immunofluorescence yielded a positive finding in 9 of 60 samples. Immunofluorescence by itself yielded a positive result in 20 cases. Thirty samples were negative by both cultivation and immunofluorescence and in one case cultivation proved more effective than immunofluorescence. The immunofluorescent technique is recommended for use in diagnostics, for screening purposes and in the prevention of infantile enteritis due to *E. coli* types.

INDIA-INK IMMUNE-REACTION FOR THE DETECTION OF ESCHERICHIA COLI TYPES ASSOCIATED WITH INFANTILE DIARRHOEA

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A comparative study with the India-ink immuno-reaction and the conventional cultural technique has been carried out to detect *Escherichia coli* types associated with infantile diarrhoea from 1000 faecal samples sent for routine examination. The India-ink immune-reaction was found to be sufficiently sensitive and in the hands of a skilled diagnostician suitable for the rapid diagnosis of *E. coli* types, and for screening the contacts in outbreaks previously verified by the cultivation method.

STRONTIUM SELENITE ENRICHMENT FOR THE IDENTIFICATION OF SALMONELLA STRAINS IN BACTERIOLOGICAL EPIDEMIOLOGICAL WORK

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To find the best method of enrichment for the isolation of different *Salmonella* serotypes three procedures were carried out with 250 test materials simultaneously, *viz.* STOKES and OSBORN's sodium selenite method, PREUSS's potassium tetrathionate method and the strontium selenite enrichment procedure. The results were as follows. Three out of 5 *S. typhi* and 27 out of 34 other *Salmonella* strains could be isolated by strontium selenite enrichment. With sodium selenite, 3 *S. typhi* and 21 other *Salmonella* strains were isolated,

while 4 *S. typhi* and 9 other *Salmonella* strains were cultured when potassium tetrathionate was used. All the 5 *S. typhi* strains could be isolated when potassium tetrathionate was used in combination with one of the selenite preparations, but the combination of sodium and strontium selenite failed to improve the result. Of the other *Salmonella* strains, 31 were isolated by the combination of strontium selenite and potassium tetrathionate, 27 by the combination of the two selenite preparations, and 25 by the combination of sodium selenite with potassium tetrathionate. A *S. lexington* and a *S. cholerae-suis* var. *kunzendorf* strain could only be isolated after strontium selenite enrichment.

MODIFIED KCN SENSITIVITY REACTION

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The essence of the modification is that instead of mixing into the culture medium, KCN is placed on filter paper into the cover of a Petri dish. Thus the culture medium can be changed according to need and the KCN sensitivity of bacteria differing from enteric bacteria in this respect can be investigated. With an appropriate culture medium, the reaction could be evaluated after 18—20 hr. Ten to 12 different strains could be inoculated onto the same plate. In the case of enteric bacteria, the results did not differ from those of the classical method. *Staphylococcus*, *Listeria monocytogenes*, and *Corynebacterium diphtheriae* also proved to be sensitive, *Streptococcus* and *Erysipelothrix insidiosa* strains were not sensitive; aerobic spore-forming strains differed in sensitivity to KCN. Beside other biochemical reactions the modified KCN sensitivity reaction may be a useful tool in bacterial diagnostics.

SUBSTITUTION OF THE CARCINOGENIC α-NAPHTHYLAMINE BY DIMETHYL α-NAPHTHYLAMINE IN THE NITRITE ANALYSIS TEST

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The nitrite analysis is widely used in bacteriological routine. In view of its carcinogenic effect, α-naphthylamine is not available in some countries. For this reason the compound was substituted by dimethyl α-naphthylamine. The latter non-carcinogenic compound can adequately be used instead of α-naphthylamine in the Ilosvay-Griess reaction.

ANAEROBIC CHAMBER FOR THE ISOLATION OF OBLIGATE ANAEROBIC BACTERIA

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An anaerobic chamber of $120 \times 60 \times 60$ cm size, made of polymethylmethacrylate was used for the isolation of obligate anaerobic rumen and enteric bacteria. The chamber supplied with gloves was filled before work with CO_2 (6 litres/min flow rate), and its continuous flow was ensured by a slight overpressure during the whole working process. CO_2, being 1.5 times as dense as air, slowly presses it out of the chamber. Therefore, dilution of the introduced sample and inoculation of media can be done under O_2 free conditions. This can be controlled by methylene blue indicator placed in the upper level of the chamber. The first phase of the anaerobic preparation of the Reinforced Clostridial Medium (prepared according to the Oxoid formula and used for culturing rumen and enteric bacteria, supplied by 5% bovine blood and 10% sterile rumen fluid) is made outside the chamber, where the medium is bubbled with CO_2 gas at a rate of 6 litres/min in 56°C water bath until the initial pH 8 has decreased to pH 7.2. In this manner, oxidation of cysteine-HCl added to the medium for the reduction of the redox potential may be avoided in the superficial layer of the culture medium. After inoculation the plates are incubated at 39°C in 10% CO_2 and 90% N_2 atmosphere for 3 days. The anaerobic chamber used is essentially similar to that of LEACH, but it has the great advantage that O_2 suction can be avoided, wherefore its use is simple and less expensive.

Virology

EBV ANTIBODIES IN SYSTEMIC LUPUS ERYTHEMATOSUS

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Serum samples from 70 patients with systemic lupus erythematosus (SLE) and those from 70 control subjects of the same age and sex distribution were tested by immunofluorescence for EBV antibodies. Both the number of subjects with high-titre anti-EBV antibodies (17 vs. 7) and the geometric mean of the titres (45.4 vs. 27.2) were higher in the SLE group than in the control group, but the differences were not significant statistically. The respective values for the anti-CMV antibodies were 10 vs. 9 and 27.1 vs. 24.3. In some SLE sera the IgG concentration was elevated, but only part of these had high anti-EBV titres and *vice versa*. The literary data suggesting that the anti-EBV titre is consistently high in SLE could not be confirmed.

TIME OF IMPLANTATION OF MAREK DISEASE VIRUS IN YOUNG CHICKENS

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Sixty-one layers were investigated in a stock highly-infected by the Marek-disease herpes virus (MDHV). All of them gave a positive agar-gel precipitation test, the geometric mean of the titres being 1 : 32. Seventy-four per cent had detectable viraemia at the time of egg-laying. Blood samples from 96-day-old chickens in this breeding were tested for MDHV without any success. Maternal antibody was detected in 76% of the chickens. Virus could not be isolated from 32 1—3-day-old chickens raised in the room in which the highly-infected layers were kept. The earliest time when the virus could be isolated from such chickens was 4 weeks after hatching. The gel precipitation test became positive after the 6th week. One hundred and twenty-eight chickens from the same breed kept in isolator up to 10 weeks of age remained negative both for virus and for antibodies in the gel precipitation test. One group of 1-day-old chickens was inoculated intraperitoneally, another group intramuscularly, with MDHV. In these chickens viraemia appeared from the 8th day on. It is concluded that the site of the entry of the virus is of importance as regards the time of implantation of the MDHV. Parenteral infection is not, infection by the natural routes is, prevented by maternal antibodies. The results present further strong arguments against the germinal transfer of MDHV.

DELAYED REAPPEARANCE OF INFECTIOUS CYTOMEGALOVIRUS AFTER METABOLIC INHIBITION BY CYTOSINE ARABINOSIDE

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Human embryonic fibroblast cultures were inoculated with cytomegalovirus (CMV) and treated with cytosine arabinoside (ara-C). In the 4—5-day period following the removal of ara-C, infectious CMV could not be recovered from disintegrates of such cultures. However, if intact cells from the same cultures were added to human embryonic fibroblast cultures, immediate virus synthesis was initiated by 1 to 2% of the cells, indicating that the virus had been carried by these cells. In 40 to 50% of the cells, the immunofluorescence test revealed virus-specific antigen filling up the nucleus diffusely. From the 4th to 5th day following the removal of the ara-C, intranuclear and cytoplasmic antigens were demonstrable in the cells and infectious virus could be isolated from disintegrated fibroblasts as well. The period from the inoculation to the

reappearance of the virus is called latent period of infection. The cells in the latent period could be superinfected by CMV or type-1 or type-2 herpes simplex virus if the cultures were inoculated with these viruses immediately after removal of the ara-C.

ANTIGENIC ANALYSIS OF PROTOTYPE INFLUENZA/A/H3N2 STRAINS BY THE IMMUNE SERUM ABSORPTION METHOD

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Prototype strains of the influenza/A/H3N2 virus can be arranged on a gradient showing the degree of the antigenic drift which the haemagglutinins of the strains have undergone. The demonstration of fine antigenic differences is based on an immune serum absorption micro-test which allows a detailed antigenic analysis of strains. The gradient provides information on variations in the strains occurring in different geographical areas and its use may be helpful in differentiating between introduced strains and locally developing variants.

SOME IRREGULARITIES OF THE 1971 INFLUENZA EPIDEMIC IN HUNGARY AND ANTIGEN ANALYSIS OF THE ISOLATES BY A SERUM ABSORPTION TEST

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In Hungary, the 1971 influenza epidemic, unlike the former influenza A2 epidemics, started in an unusually early season and seemingly in two foci. The average morbidity rate (19.3%) was in the usual range but the geographical distribution of the cases was irregular. One hundred and seventy-nine isolates were analysed antigenically with monospecific sera prepared by serum absorption. They represented two well-distinguishable variants of the influenza/A/H3N2 virus: 126 strains were closely related to the prototype strain Hong Kong/1/68, and 33 strains to the strain England/878/69. It is suggested that the variants had developed locally and were not introduced. Absorbed sera monospecific for closely related variants, like those applied in the present work, are recommended for the rapid identification of influenza virus isolates.

DIAGNOSIS OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTIONS BY THE FLUORESCENT ANTIBODY METHOD

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The fluorescent antibody method has been simplified by using infected cell cultures freeze-dried on slides. This method allows standardization of the test. Two-hundred patient sera have been tested in parallel with this method and the complement fixation test. The fluorescent antibodies appear earlier than the CF antibodies, in the neural phase of the illness already. The procedure is quick and specific.

INVESTIGATION OF ARBOVIRUS NATURAL FOCI IN GYŐR-SOPRON COUNTY, HUNGARY

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Investigation of natural foci of TBE virus in the Mosonmagyaróvár and Sopron regions was continued between October, 1970 and October, 1971. Information has been collected on the reservoirs and vectors of virus as well as on the presence of antibodies against TBE and some other arbovirus strains in human and in bovine sera of different stages. More than 5000 ticks of different stages were collected in the woods for virus isolation; 98.7% of them proved to be *Ixodes ricinus;* 0.4% *Haemaphysalis concinna;* and 0.9% *Dermacentor marginatus*. From three pools of *Ixodes ricinus nymphae*, 3 TBE virus strains have been isolated: 2 in Tómalom, 1 in Fertőboz. Infestation of *Rodentiae* by ticks in the same regions was frequent. From the blood of one *Clethrionomys glareolus* an unidentified non-TBE arbovirus strain was isolated. Serum samples from 11 of 246 small mammals contained HI antibodies against TBE. Twelve of the 246 proved to be positive in CF test against UUK-Potepli-63 and 2 of 97 against TBE virus. The frequency of antibodies in human and bovine sera was established with different arbovirus antigens in HI, CF and/or neutralization tests. Of 366 human sera 20 were found positive against TBE, 15 against WN and 7 against UUK-Potepli-63 virus. The respective data for 100 bovine sera were 3, 9 and 1. Forty-one bovine sera contained anti-CLV antibodies.

EXAMINATION OF LYMPHOCYTES FROM CHILDREN WITH APPENDICITIS

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Lymphocyte transformation tests and immunofluorescence investigations were carried out with lymphocytes of children suffering from appendicitis associated with mesenteric lymphadenitis. Peripheral lymphocytes from 39 out of 46 patients appeared to be sensitized by adenovirus 1 and/or 6 antigens of the viruses which had been isolated from similar cases. Lymphocytes from 40 patients were examined by immunofluorescence, too. Fluorescence suggestive of the presence of viral antigen was demonstrated with anti-adenovirus 1, anti-adenovirus 12, and anti-herpes simplex conjugate in 19, 16 and 5 cases, respectively. Cytological preparations from 8 surgically removed appendices showed fluorescence with anti-herpes simplex conjugate in 6 cases and with anti-adenovirus 1 conjugate in 4 cases (3 specimens proved positive with both conjugates). Cases with purulent, gangrenous appendicitis served as controls. In these cases neither sensitized lymphocytes nor positive immunofluorescence could be demonstrated. The present data have confirmed the previous observation suggesting that viruses may play a role in nonpurulent appendicitis associated with lymphadenitis.

INTERFERON INDUCTION AND IMMUNOSUPPRESSION BY ADENOVIRUSES IN CHICKENS

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Chickens inoculated with human adenoviruses are producing interferon, which appears in the blood 2 to 4 hr after intravenous inoculation and disappears by the 96th hr. The primary antibody response of the inoculated chickens to sheep erythrocytes was found to be blocked in the period from the 3rd to the 16th day after inoculation. In this period the lymphocytes obtained from the spleen of the chickens showed a reduced transformation rate when treated with phytohaemagglutinin. It is supposed that the produced interferon has a role in the induction of immunosuppression.

REGULATION OF INTERFERON PRODUCTION IN VITRO AND IN VIVO BY INFLUENCING THE ADENYLATE CYCLASE SYSTEM

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It has been studied whether cellular resistance and production of IF can be influenced *in vitro* and *in vivo* by (i) addition of exogenous cAMP or (ii) activators of adenylate cyclase, *viz.* isoproterenol, prostaglandin E_1 (PGE_1), DEAE-dextran; (iii) stabilization of intracellular cAMP formed by inhibitors of phosphodiesterase such as theophyllin, and whether (iv) adenylate cyclase can be activated by IF. Cellular resistance was enhanced *in vitro* and the production of IF was increased 5 to 10 times at appropriate concentrations of the drugs; above an optimum concentration, all drugs were inhibitory. Appropriate concentrations of PGE_1 increased the level of IF 5 to 10 times *in vivo*, or potentiated the effect of inducers given in suboptimal amounts. Higher concentrations of PGE_1 decreased the IF level. Activation of the adenylate cyclase system in L cells by crude mouse IF preparations was tested by determining the amount of radioactive cAMP formed. IF treatment increased the level of cAMP 1.8 to 2.2 times. Potentiation of IF production *in vitro* and *in vivo* — either with IF (priming), or by using drugs increasing the cAMP level (activator) — is probably brought about by the same mechanism, *i.e.* an activation of the adenylate cyclase system.

INTERFERON, TOTAL RNA AND PROTEIN SYNTHESIS IN MOUSE SPLEEN CELLS AFTER ACUTE GAMMA IRRADIATION

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Interferon, nucleic acid and protein synthesis was investigated in spleen cells of BALB/c mice irradiated with gamma rays (400 R). After induction by poly I : C the production of interferon by spleen cells increased 8 to 16 times in the irradiated animals as compared to non-irradiated controls. Such an increase occurred in cell suspensions containing 70% macrophages as well as in pure lymphocyte suspensions. Using Newcastle disease virus (NDV) as inducer, the production of interferon in non-irradiated and irradiated mice was accomplished mainly by cell suspensions containing macrophages. Six and 18 hours after the injection of poly I : C or NDV, a 60% activation of RNA synthesis ([^{14}C] uridine incorporation) was observed in the spleen cells

of intact mice, but the synthesis of total protein ([^{14}C] amino acid mixture incorporation) was not activated. In the spleen cells of irradiated mice, RNA and protein synthesis was reduced which showed a further decrease after injection of poly I : C or NDV, in spite of the increasing interferon production.

THE ROLE OF INTERFERON IN THE INTERFERENCE WITH VARIOUS VIRUSES

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Reproduction of avirulent NDV and Sindbis virus strains is inhibited by both living and inactivated avirulent NDV strains. The interference with the Sindbis virus, a virus highly sensitive to interferon, can be attributed to the interferon, whereas in the interference between the NDV strains the interferon plays no role. Interferon can only be considered a mediator of interference if the inhibited virus is highly sensitive to it.

VIRUS VIRULENCE AND INTERFERON

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The interferonogenic capacity of NDV, infectious bronchitis virus (IBV) and Aujeszky virus (AyV) strains was studied comparatively to show whether interferon had a role in virus virulence. In some systems (cell cultures, chicken embryos or chickens) infected with NDV or IBV, the more interferon was produced the more intensive was the multiplication of the given virus. The avirulent AyV strains proved to be better interferon-inducers and 5 to 10 times more sensitive to interferon than the virulent strains. However, the late appearance of interferon contradicts its role in the low virulence of the said strains.

AETIOLOGICAL ROLE OF ENTEROVIRUSES IN INFECTIOUS DISEASES OF CHILDREN

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The occurrence of enteroviruses was investigated in children from 0 to 14 years of age in Hajdu–Bihar county in the years 1969—1971. Excretion rate was the highest at 2 to 3 years of age; the seasonal peak was in the months

from June to August. From patients with abacterial meningitis, echovirus 6, 9 and 30 strains; from herpangina cases, coxsackie B3 and B4 strains; from upper and lower respiratory illness, coxsackie B3 and B4 strains; from grippal gastroenteritis cases, coxsackie B2 strains, were isolated the most frequently.

EFFECT OF SOME FACTORS ON THE BIOLOGICAL ACTIVITY OF MEASLES VIRUS (STRAIN L-16)

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The demonstrability of the L-16 vaccine strain of measles virus in cultures of the III/1 rhesus monkey cell line was not influenced by the age of the cells, the number of cells per tube, the size of the inoculum between 0.1 and 1.0 ml, and the serum content of the maintenance fluid. If, however, proteinless Hanks' solution of Parker's No. 199 enriched with bovine amniotic fluid was used as maintenance fluid, or the tubes were incubated for 10 to 60 min between the inoculation and the addition of the maintenance fluid, the cell cultures were sensitive. The best results were obtained when the inoculated cultures were incubated at 34°C, using Parker's No. 199 as maintenance fluid.

ROLE OF RIBONUCLEIC ACID AND CELL MEMBRANE IN THE INCORPORATION OF NEUTRAL RED BY TISSUE CULTURE CELLS

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Neutral red (3.5—5.0 $\mu g/cm^2$) was shown to be taken up by permanent monkey kidney cell monolayers under usual conditions. Intensive staining of the cytoplasm and photosensitization of RNA viruses suggested the role of single-stranded RNA in dye binding. Pronounced changes in the optical absorption spectrum of neutral red were observed *in vitro* at 530 nm in the presence of commercial yeast RNA. Gel filtration experiments and spectrophotometric data suggested that the ratio of bound dye and nucleotides was always less than 1 : 55 and polynucleotide chains consisting of less than 30 nucleotides did not bind measurable amounts of neutral red. Inhibition of dye uptake was found in the presence of yeast RNA solutions. Neutral red was released from stained cell monolayers on DMSO, cysteine or RNA treatment, without loss of cell-viability. On the basis of these results the important role of the cell

membrane and a minor importance of cell RNA in neutral red incorporation is suggested. The observed phenomena may provide a tool for experiments on virus–cell membrane interactions.

EFFECT OF ADENOVIRUS INFECTION ON THE PRODUCTION OF C4 INACTIVATOR BY KB CELL CULTURES

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It had been shown previously that in uninfected KB cell cultures an anticomplementary substance identical with activated C1 esterase is produced, which then inactivates the C4 component of the complement. The C4-inactivating activity of the medium of KB cell cultures is increased by infection with the adenovirus 3, 4, 11 or 13 prototype strains. Infection with the adenovirus 1, 2, 5, 6, 7, 12 or 14 prototype strains, on the other hand, causes hardly any or no increase in this anticomplementary activity. Strains isolated from patients behave similarly to the respective prototype strain. No relationship could be demonstrated between the C4-inactivator-inducing capacity of adenovirus strains and their capacity to cause early cytopathic changes.

THE DYNAMICS OF THE SERUM IMMUNE GLOBULIN CONCENTRATION FROM LATENCY TO LATE CONVALESCENCE IN HEPATITIS PATIENTS WITH OR WITHOUT HEPATITIS B ANTIGENAEMIA

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Blood was taken from 219 patients suffering from serum hepatitis soon after the supposed time of infection and subsequently on 17 occasions. The last samples were taken 18 months after clinical recovery. Hepatitis B (Australia) antigenaemia was demonstrable in one or more samples of 187 patients. The serum IgM and IgG levels of these patients showed a well-defined peak late in latency and in convalescence, respectively; the IgA values remained below the normal level during the whole period. In the blood of the 32 hepatitis B-negative patients each of the three immunoglobulins reached an elevated value once during the observation period. Thus, the behaviour of the serum IgA level allows to distinguish between hepatitis B-positive and negative cases of serum hepatitis.

INVESTIGATION OF THE ROLE IN ACUTE AND CHRONIC HEPATITIS OF THE ANTIBODY AGAINST THE TWEEN-80-TREATED HEPATITIS B ANTIGEN

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Preparations rich in Dane-particles 40 to 70 nm in diameter were treated with Tween-80 as described by Almeida *et al.* The preparation thus obtained was examined by electron microscopy, and used in CF tests as antigen. Antibodies to this antigen were demonstrated in serial serum samples of 30% of 136 patients with acute hepatitis during the clinical illness and in only 10% of 371 convalescents. In contrast, antibody was found in the serum of nearly 60% of the 74 patients with chronic hepatitis. There was no correlation between the time of recovery and the appearance of serum antibody. These results call attention on the possible important role of Dane-particles, particularly of their inner structure as antigen, in the pathomechanism of viral hepatitis. The assumption of Almeida *et al.* that the antibody against the inner structure of the Dane-particles had a role in the recovery from hepatitis could not be confirmed.

SCREENING OF MOTHERS AND HEALTHY MATURE NEWBORNS FOR HEPATITIS B ANTIGEN

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Blood samples were taken from 228 mothers and from their healthy mature newborn babies (umbilical cord blood). The samples were tested for hepatitis B (Australia) antigen by immunoelectrophoresis. All the newborn samples proved to be negative, whereas two mothers had antigenaemia. These data are compared to results obtained by the authors from screening of premature children and blood donors.

HEPATITIS B ANTIGENAEMIA IN OUTPATIENTS OF CHILDREN'S HOSPITALS

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Blood samples from 400 unselected outpatients 3 to 17 years of age were tested for hepatitis B antigen using the immunoelectrophoresis method. Five children proved to be positive. Hardly any connection could be supposed between their history and the antigenaemia.

ATTEMPTS TO IMMUNIZE SOWS AGAINST TRANSMISSIVE GASTROENTERITIS WITH A LIVE VACCINE

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The relationship between the antibody level in the milk of affected sows and the end of the epizootic was investigated, during a natural transmissive gastroenteritis (TGE) epizootic. The epizootic ceased when the antibody had appeared in the milk and in the colostrum. Subsequently oral vaccination with an attenuated live vaccine was carried out in large swine stocks susceptible to TGE. Neutralizing antibodies were determined in serial blood and colostrum samples from sows vaccinated once or twice. The antibody level in the colostrum was approximately equal to the actual blood level. The antibody level produced by two vaccinations at an interval of six weeks approximated the level found during the epizootic. The high titres could be restored by a single revaccination 6 months after the vaccination experiment. The antibody levels in the colostrum and in the milk of the vaccinated sows were sufficient for ensuring a firm immunity to suckling piglets in a natural epizootic.

VIRAL POLLUTION OF RECREATIONAL FRESH-WATERS IN HUNGARY

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A total of 287 surface water samples were examined virologically in the years 1969—1971. The Lake Balaton, the Lake Velence and the Danube Bend were regularly sampled, *i.e.*, during the recreational season, from May to September. One sample each of the Lake Balaton and the Danube, and six

samples from the Lake Velence yielded virus. Samples from the Danube and the Tisza — taken far from bathing areas — were found more contaminated, resulting in the isolation of eleven virus strains. Of the isolates, six were identified as reovirus type 1, the others proved to be echovirus types 1, 6, 7, 8, 11 and 14, and poliovirus type 1.

SENDAI VIRUS INDUCED FUSION OF HUMAN AND CHINESE HAMSTER CELLS

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Human fibroblasts and Chinese hamster glyD mutant cells were fused in the presence of 300 HAU UV inactivated Sendai virus. The hybrids were selected in Ham's F12 gly^- medium, containing 10% foetal calf serum macro fraction. After 10 generations, chromosomal analysis showed 8 human chromosomes persisting in the hybrid cells. The applied technique is a reproduction of the previously described methods which proved useful in mapping human chromosomes and studying gene expression in hybrid cells.

TRYPSINIZATION OF ORGANS WITH THE AUTOMATIC APPARATUS "TRIPSZIMAT"

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Tripszimat (Labor MIM, Budapest), a closed system, can be used for the separate disperging of cells, simultaneously from three organs. Experience with trypsinization of rhesus-monkey, guinea pig and Syrian hamster kidney cells and human and chicken embryos is presented. At a flow rate of 80 drops/min and continuous removal of the cell suspension, the cell release curve reached its peak between the 40th and 90th minutes of trypsinization; the number of disperged cells increased with increasing temperature — the temperature optimum was 33° to 36°C, at higher temperatures the cells were impaired in viability; cell viability was not influenced by the presence or absence of divalent cations in the trypsin solution or in the suspending solution of cells; the obtained cell number/g tissue was, on the average, 2 to 2.5 times as high as that obtained by the conventional (Young) method. These conclusions were drawn from 53 trypsinization procedures and have been confirmed by a 3-year routine.

Immunology

DETECTION OF SENSITIZING SUBSTANCES IN VIRAL VACCINES

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The guinea pig sensitization test has been applied for the detection of residual serum protein in viral vaccines produced in tissue culture. Guinea pigs of 250 g body weight were injected with one human dose of vaccine each and challenged by an intravenous injection of 1 ml bovine serum one week later. Injecting 10 animals with each preparation, serum dilutions as high as 1 : 100, 000—1 : 500, 000 could be detected by this method. Measles, mumps, rabies and rubella vaccines and inactivated smallpox antigens produced in different countries, 36 batches altogether, have been tested by the method; 23 batches proved to contain in the human dose an amount of bovine serum sufficient for sensitizing a guinea pig.

REACTIONS OF INFANTS TO LIVE MEASLES VACCINE

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The vaccines applied in the first part of the work contained, on the average, 550 $TCID_{50}$/dose of the Leningrad 16 strain. The vaccinations were carried out in a period when the evaluation of the reactions was not disturbed by natural infection. The reaction of 2572 infants 9 to 12 months of age was observed 6 to 14 days after vaccination. Local reaction was observed in 0.23%, fever in 18.9% (>39.5°C in 1.56%); rash in 6.7%; catharrhal symptoms in 1.3%; malaise in 1.1%. Three weeks after vaccination blood was taken from 605 infants to titrate measles antihaemagglutinin. The geometric mean (g. m.) of the titres was 1 : 97.5. A positive correlation was found between the febrile reaction and the HI antibody response. Two years after the vaccination the g. m. for 82 children was 1 : 36, and 97.6% of these children had an HI titre 1 : 4 or higher. Three years after the vaccination the respective values for 50 children were 1 : 5.3 and 78%. Twenty-two children 9 to 21 months of age were immunized with a vaccine containing 350 $TCID_{50}$ of virus. These children showed weaker reactions as manifested in fever and exanthem, and a weaker antibody response (g. m. 1 : 86 for 19 children) than the 38 children vaccinated with 700 $TCID_{50}$ (g. m. 1 : 168 for 27 children).

SEROLOGICAL EVALUATION OF IMMUNIZATIONS WITH INACTIVATED MUMPS VACCINE

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$Al(OH)_3$-gel-adsorbed formalin-inactivated vaccines were prepared from mumps virus grown in embryonated eggs. The eggs used in the production of vaccines No. 1 and No. 2 had been laid by hens previously immunized and non-immunized, respectively, with live NDV vaccine. Both vaccines contained 1000 HA U/ml. In animal experiments vaccine No. 2 induced a significantly more marked immune response than did vaccine No. 1. Children 2 to 5 years of age were immunized with 2×0.5 ml of vaccine No. 2 at an interval of 6 weeks. The postvaccination HI titres were as high (geometric mean, 1 : 20.0) as the mumps convalescence titres of children of the same age (1 : 20.5).

EXPERIMENTS WITH THE "SAD-VNUKOVO" RABIES VACCINE VIRUS STRAIN

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Experimental vaccine was prepared from the "Sad-Vnukovo" strain grown in tissue culture and tested in albino mice, guinea pigs, rabbits, dogs and sheep. The vaccine proved to be innocuous when inoculated intracutaneously and intramuscularly. After intracerebral inoculation the mice became ill from the 5th day on, and all had died by the 16th to 18th day depending on the vaccine dose. Of the inoculated rabbits 50% died, the others survived without any reaction; the intracerebrally inoculated sheep remained healthy. The potency of the experimental vaccine was comparatively examined with a commercial phenol-glycerolated vaccine prepared from sheep brains infected with the Hőgyes fix virus. The Habel test and the virus neutralization test were used. The experimental vaccine proved to be at least as effective as the commercial vaccine.

PATHOGENICITY OF A MAREK DISEASE HERPES VIRUS STRAIN DURING SERIAL PASSAGES IN DUCK EMBRYO FIBROBLAST CULTURES

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Virus isolated from chickens with acute Marek disease was carried over serial passages in duck embryo fibroblast cultures. The strain was tested for pathogenicity in highly susceptible chickens after 13 and 40 passages. All the

chickens infected with the 13th passage-level virus developed virus-induced tumours and died. Of those inoculated with the 40th passage-level virus one quarter survived. In one quarter of the died chickens, tumour could not be detected even histologically. It is concluded that in the course of passage in cell cultures the acute pathogenicity of the virus decreases at a slower rate than does its oncogenic capacity.

IMMUNITY TO SMALLPOX

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In order to determine the titres of neutralizing antibodies, blood samples taken from 347 individuals were tested by the vaccinia plaque reduction test in tissue cultures. Smallpox vaccine given at one year of age provoked a strong response. In the 1—4-year-age group, 75% of the children tested had demonstrable antibodies. In the next two groups (5—7 years and 8—11 years) the rate of positivity was 53% and 40%, respectively. Despite the compulsory vaccination at 12 years of age, in the 12—14 year age group only 54% of the children had plaque-reducing antibodies. The percentage was not much lower (42%) above 40 years.

IMMUNOCHEMICAL ANALYSIS OF THE SERUM FRACTIONS OF MICE WITH RAUSCHER-LEUKAEMIA

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Soluble antigens of α_2 and β_2 mobility are demonstrable by immunoelectrophoresis in the sera, made free of virus, of mice with Rauscher-leukaemia. It was proved by indirect membrane immunofluorescence that the antigens are identical in specificity with the surface antigen(s) appearing in the course of the cell transformation. It is supposed that the soluble antigen is identical with the tumour-specific antigen of the Rauscher-leukaemia cells, for the whole antibody contents of the sera of both the mice immunized with inactivated Rauscher virus and those in remission from Rauscher-leukaemia are absorbable with it. In the serum of infected DBA/1 mice (a strain moderately susceptible to the Rauscher virus) antigen–antibody complexes are present from which the antibody can be separated at pH 2.4. As remission ensues, the ratio of free antibodies to bound antibodies gradually increases. The soluble antigens, due to binding of cytotoxic antibodies, may promote the progression of leukaemia.

COMBINED EFFECT ON THE CELLULAR IMMUNE RESPONSE TO LYMPHOCYTIC CHORIOMENINGITIS VIRUS OF SUBSTANCES ALTERING THE CELLULAR IMMUNE RESPONSE OF MICE

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Six-week-old Swiss mice (20 mice per group) were injected intraperitoneally with phytohaemagglutinin (PHA, 0.25 mg per mouse), pertussis vaccine (9×10^9 live germs per mouse) and/or intravenously with dianhydrodulcite (DBD-epoxide, 11×0.1 LD_{50} per mouse). The mice were then infected intracerebrally with 100 LD_{50} of the WE strain of LCM virus. The subsequent neurological symptoms characteristic of the immune response to LCM virus infection, the time of death of the animals as well as their spleen weight were registered. Reisolation of LCM virus on the 21st day from the brains of the surviving mice was successful in each case. The immunosuppressive effect of DBD-epoxide (causing lymphoid atrophy) was influenced in a different way by PHA and the pertussis vaccine: (i) DBD-epoxide + pertussis vaccine induced a more pronounced depression of the cellular immune response than either of the two agents separately; (ii) when DBD-epoxide was combined with PHA, no immunodepressive effect of the former could be observed.

THE ROLE OF MACROPHAGES IN ANTIBACTERIAL IMMUNITY

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Anti-macrophage serum (AMS) was produced in rabbits by immunizing them with murine peritoneal cells. The serum had an agglutinin titre of 1 : 2187 and a cytotoxicity titre of 1 : 3645. Mice were infected intraperitoneally with this AMS. In the subsequent 24 hr period the number of living macrophages in the peritoneal fluid of the mice fell to 0 or 4—5% of the initial number. When mice immunized by sheep erythrocytes or infected by *Vibrio cholerae* were treated with the AMS, no immunosuppressive effect ensued. On the contrary, the immune response to the sheep erythrocytes was slightly stimulated, the resistance to *V. cholerae* was significantly increased.

TUBERCULIN SENSITIVITY OF SENSITIZED LYMPHOCYTES

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The sensitivity of lymphocytes of guinea pigs inoculated with live *Mycobacterium bovis*, *M. avium*, *M. ulcerans* or *M. xenopei* cultures to protein and polysaccharide antigens of different mycobacteria has been studied. The lymphocytes of guinea pigs sensitized with *M. bovis* reacted to the bovine and avium antigens with blastic transformation in 39 and 27%, respectively. In the group infected with *M. avium*, the respective percentages were 22 and 48%; in the *M. ulcerans* and *M. xenopei* groups somewhat lower values were obtained. It was only in the group infected with *M. bovis* that the polysaccharide antigen produced from *M. bovis* AN_5 strain induced blastic transformation; the lymphocytes of the other groups gave no secondary immune response to this antigen. The experiments were completed by immunofluorescence. Fixed samples of cell cultures treated with bovine protein antigen for 24, 48 or 72 hr were incubated with FITC conjugated immunoglobulin produced from rabbits immunized with *M. bovis*. The results were consistent with those presented above.

AN ANALYSIS BY MODERN COMPUTATION TECHNIQUES OF THE CORRELATION BETWEEN BLOOD GROUP AND EGG YIELD

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The correlation between phenotypes within the two most important group systems (A and B), on the one hand, and egg yield and egg weight, on the other, were analysed for 1210 hens. The phenotypes were determined by the haemagglutination test. The quantitative characteristics of production (number of eggs laid per 3 1/2, 6 and 11 months; egg weight) were grouped using two-rowed marginal punched cards, according to phenotype. The data thus obtained were subjected to mathematical-statistical analysis. Significance levels were determined by variance analysis and the phenotypes were ranked by means of Duncan's multiple range test. The results of the statistical analysis may be summarized as follows. As to egg yield (i) there are positive (10/10, 5/5, 5/13, 2/5) and negative correlations (2/13, 1/10, 1/1, 5/10, 2/10) between some phenotypes within the blood group system B and the start of egg laying; (ii) there are positive (B2/5 + A2/2, B5/5 + A3/5, B2/5 + A1/2) and negative correlations (B5/10 + A2/3, B1/2 + A1/1, B1/1 + A1/1) between some combinations of system-A and system-B phenotypes and the egg yield. As to egg weight, some positive and negative correlations within system A

proved to be significnat. Similar correlations could not be demonstrated in system B. The direction of the correlations was consistent during the period under analysis. The authors suppose that the results will be useful in the early selection of breeding stocks.

PRODUCTION OF ANTITOXIC TETANUS SERUM IN HORSES, USING CONCENTRATED PURIFIED ANTIGENS

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The immune response of horses has been studied during hyperimmunization with purified concentrated tetanus antigen of different types. The preparation method of the antigens and the correlation between their dose and the antitoxic response are presented. Fluctuation of the serum protein fractions was also determined. Only purified concentrated antigens proved to be suitable for the production of tetanus antitoxic hyperimmune horse sera.

ISOLATION OF ANTI-TETANUS ANTIBODIES ON THE BASIS OF IMMUNOLOGICAL SPECIFICITY

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The specific antibodies were isolated from human anti-tetanus hyperimmune plasma, as well as from horse, cattle and sheep anti-tetanus sera, using an immunoadsorbent prepared from tetanus toxoid. The isolated immunoglobulins were examined by physicochemical and immunochemical methods, and their sensitizing effects were compared to those of crude sera.

STANDARDIZATION OF THE TOXICITY TESTS FOR PERTUSSIS VACCINES AND COMBINED VACCINES INCLUDING PERTUSSIS COMPONENT

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According to the requirements of WHO and the prescriptions of several Pharmacopoeas, pertussis vaccines must be subjected to toxicity tests. The detailed requirements are, however, different. The results of the toxicity tests

with pertussis vaccines are determined, first of all, by the method of their production and the sensitivity of the mouse strain used. In *Bordetella pertussis* strains there are several different antigenic components (thermostable lethal toxin, endotoxin HSF and LSF) which may cause changes in the body weight of mice. Different *B. pertussis* strains may contain these antigens in different proportions and different mouse strains vary in sensitivity to these antigens. It seems necessary to standardize both the mouse strains and the bacterial strain. Using appropriately selected mouse strains, the most severe requirements of Pharmacopoeas can be fulfilled. In the authors' opinion, the toxicity tests offer little information on the reactivity of vaccines in man.

REVISION OF THE DI-PER-TE REVACCINATION DOSE IN HUNGARY

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In Hungary, the basic immunization of infants against diphtheria, pertussis and tetanus as well as the revaccinations of 3-year-old children is carried out with a vaccine containing 15 Lf diphtheria toxoid, 5 BU tetanus toxoid, and 15×10^9 *Bordetella pertussis* per dose, all adsorbed to aluminium phosphate gel. The revaccination dose was repeatedly revised: the postvaccination antibody titres were determined in groups consisting of 41 to 51 children each. It was found that the respective dose can be reduced to 7.5 Lf, 2.5 BU and 11.5×10^9 without any immunological disadvantage.

Miscellaneous subjects

PURIFICATION OF STAPHYLOCOCCAL ENTEROTOXIN

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Purification of staphylococcal enterotoxin A and B was carried out by CM-cellulose cation exchange chromatography. The fractions were controlled by specific monovalent (Bergdoll) sera and by nonspecific sera obtained from rabbits immunized with the filtrate of different staphylococcal strains. The presence and the quantity of the toxin were tested by the specific serum and the purity of the fractions by the nonspecific serum. Seventy gradient filtrations, 435 immune electrophoreses and 3000 micro-precipitations were carried out in the course of the experiments. Staphylococcal strain No. 262 was the most appropriate for enterotoxin B production. This toxin could be purified

on one column. The crude enterotoxin A of the FRI-100 strain yielded a pure fraction only after a further CM-column purification of the double-controlled fractions obtained by gradient filtration. The control results have been demonstrated on original preparations.

IDENTIFICATION OF MYCOPLASMA

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The present studies were initiated in common with the Veterinary Research Institute of the Hungarian Academy of Sciences. The experiences are as follows: (i) single and grouped human mycoplasma infections are not rare in Hungary; (ii) mycoplasma infections were usually unrecognized and treated *ex juvantibus* with antibiotics; (iii) only patients in a dangerous condition were further examined and the diagnosis was established only in these cases; (iv) a local outbreak of mycoplasmosis with a fatal and a severe case was recognized. According to the experience obtained, examinations carried out in due time ensure the proper diagnosis and adequate treatment of mycoplasmoses.

EXAMINATION OF ACHELOPLASMA STRAINS ORIGINATING FROM PIGS

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After selection, 10 *Acheloplasma* strains isolated from different organs of pigs were investigated in respect of their resistance and biochemical and serological properties. (i) Two strains belonged to the *A. axanthum* species. The occurrence of this species in animals has been established for the first time. (ii) Seven strains could be ranged into the *A. laidlawii* species, but they differed from reference strains *A. laidlawii* A. PG 8 and *A. laidlawii* B. F-8 in serological properties and metabolical reactions, namely 3 in failing to hydrolyse esculin, 2 in breaking down cellobiose, 2 in both of these activities as well as in the degree of serological reactions. (iii) One of the 10 strains differed from every known *Acheloplasma* strain in all the applied tests.

PROTOTHECA, A POTENTIALLY PATHOGENIC GENUS OF ACHLORIC ALGAE

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Yeast-like, achloric alga strains were isolated from the slimeflux of trees, samples of sewage-treated plants and human clinical material. Some of the strains could be classified into the species *Prototheca trispora*, *P. zopfii*, *P. moriformis*, *P. wickerhamii* or *P. ubrizsyi;* the others could not be identified with any valid species of the genus *Prototheca*. Attention is drawn to this group of potentially pathogenic algae living in the environment of man and animals.

CORRELATIONS AMONG RESULTS OF BIOCHEMICAL TESTS IN MICROBIOLOGICAL IDENTIFICATION, THEIR TAXONOMICAL AND PRACTICAL SIGNIFICANCE

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The number of physiological and biochemical tests applied for the identification of microbes is continually increasing. Consequently, statistical and other mathematical methods are also frequently applied. The great number of tests may be superfluous if they yield correlating results. Results of carbohydrate utilization tests applied in yeast taxonomy were analysed for correlations in the case of the most important 10 out of 40 carbon sources. The study covered the data of about 2000 strains of 350 species. Double as well as multiple correlations were demonstrated and the biochemical background has been discussed.

THE INDIA INK IMMUNO-REACTION IN MYCOLOGY

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The morphological and physiological-biochemical identification of yeast strains is laborious and time-consuming. The strains' close antigenic relationship hinders the effective use of serological methods. For overcoming these difficulties the India ink immuno-reaction was developed from the earlier

elaborated rapid immunofluorescence test. The procedure allows the diagnosis of *Procandida albicans* (the most frequent pathogen among yeasts in Hungary) in 10 minutes. The specificity of the reaction was ensured by serial dilutions of the sera instead of absorption with related species. Evaluation was improved by counter-staining of smears by safranine. The method was further developed for direct serological diagnosis of candidiasis caused by *P. albicans* and for the histological demonstration of the latter.

SIGNIFICANCE OF BEDSIDE CULTURAL EXAMINATION IN THE HOSPITAL NURSERY FOR THE RAPID DEMONSTRATION OF PROCANDIDA ALBICANS INFECTIONS AND OF CANDIDIASIS ENDANGERMENT

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The yeast flora and, especially, the occurrence of *Procandida albicans* were examined in samples from the vagina and milk of mothers and the oral mucous membrane and faeces of newborn infants at the obstetric ward and the maternity unit of two hospitals in Budapest. Pagano-Levine-Trejo type, triphenyltetrazolium chloride-containing culture medium was used. The results suggest the importance of iatrogenic infections (manual and instrumental) rather than that of the natural (vaginal) route. This makes it possible to prevent early infections. The mothers' treatment with antibiotics also increased the occurrence of late candidiasis and the development of endemics. The compulsory introduction of the above investigations is suggested.

TREPONEMOCIDE EFFECT OF CATTLE SERUM IN RELATION TO AGE

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An earlier study of treponema-resistant animals' blood sera revealed the presence of treponemocide substances in cattle serum. The treponemocide effect of cattle serum was examined following birth, after sucking colostrum, at the age of six weeks and six months. According to the results the treponemocide substance appears approximately at the age of six months.

RAPID ANTISTREPTOLYSIN LATEX-AGGLUTINATION SLIDE TEST

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During the last 3 1/2 years the number of antistreptolysin (ASL) titre determinations reached 2072 in the Laboratory of the 22nd District Polyclinic. The number of examinations showed an increasing tendency, the index numbers for the last four years were 100, 127, 142 and 160, respectively. This was the reason for the introduction of the rapid ASL latex-agglutination slide test. Its results have been compared to Böszörményi's tube-test and to its modification elaborated by Bozsóky using Takátsy's microtitrator. The commercial reagents were (i) ASL latex-reagent; aqueous suspension of polystyrol particles carrying streptolysin O. (ii) Streptolysin O: standard lyophilized haemolysin prepared from β-haemolytic *Streptococcus* . As the first step, a given quantity of streptolysin O is added to the serum on a slide. Agglutination occurs only if the ASL content of the serum is above 200 IU/ml. Dilution and inactivation of the serum can be avoided. Fifteen minutes are needed for the completion of neutralization and 4—6 minutes for the agglutination. The test is specific.

EFFECT OF PHENOXY-ACETIC ACID DERIVATIVES ON DIFFERENT BACTERIAL STRAINS

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Comparative laboratory examinations have been carried out in 66 bacterial strains belonging to 28 species (soil bacteria and bacteria pathogenic for plants, animals and man) with herbicide preparations (2,4-dichloro-phenoxy-acetic acid; 2,4,5-trichloro-phenoxy-acetic acid; sodium salt of 2,4 dichloro-phenoxy-acetic acid; 2,4,5 trichloro-phenoxy-acetic acid isoamyl ester) of analytical and technical grade. Resistance of the examined strains to the above preparations has been determined.

GROWTH KINETICS OF SOME CELLULOLYTIC MICROORGANISMS

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The growth of the strains *Cellulomones* sp. ATCC 21399, *Torulopsis utilis* 81 and 82, *Candida utilis* 147 has been studied on agar plates containing 1% carboxy-methylcellulose as a sole carbon source. All the strains formed colonies with this substrate at 30°C within an incubation period of 7 days. Fermentations were carried out with substrates prepared from milled paper, straw and corn-cob, completed with nutrients proposed by Han. Cellulose-containing materials were pretreated with 1.5% NaOH solution at 100°C; 100 ml samples were fermented in an eccentric shaker at 30°C; 10^6/ml initial cell density was used in triplicate. Cell density in the function of fermentation time was determined by direct cell counting. The exponential phase of growth was analysed by statistical mathematical methods. All the strains had reached a 10^8/ml cell count within a 24-hour fermentation period.

ISOLATION OF MYCOPLASMA PNEUMONIAE IN HUNGARY

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Mycoplasma pneumoniae was isolated from the sputum of three hospitalized patients suffering from pneumonia of protracted convalescence. Growth and metabolism of the strains was inhibited by the patients' convalescent sera. The patients lived in different areas of Hungary. In the environment of one of the patients, serum samples from 16 subjects were examined; 6 sera proved to contain antibodies to *M. pneumoniae*. Four of the positive subjects had been ill with pleuropneumonia before blood sampling.

Food bacteriology

DEMONSTRATION OF NONPATHOGENIC CONCOMITANT MICROBES IN FOODSTUFFS

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The necessity of the examination for nonpathogenic concomitants of foodstuffs is discussed first of all from the point of view of their qualitative deterioration and the reduction in their biological value, with special regard

to preparations of plant origin. In addition to the usual group methods (total germ count, yeasts, moulds, heat tolerants, etc.) some supplementing methods (*e.g.*, demonstration of lipolytic bacteria and mould toxins) are mentioned, which might be introduced later. Several specific tests (*e.g.*, demonstration of *Bacillus mesentericus* spores in flour and of *Leuconostoc mesenteroides* in sugar) and several rapid and micro-methods for screening tests or identification of microorganisms are reviewed.

DETERMINATION OF TOTAL VIABLE BACTERIAL COUNTS

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Different methods of total viable bacterial count estimation have been compared along with the effect on the estimation of different nutrients. The most appropriate dilutions of the test material in the suspending medium seemed to be 1 : 4 and 1 : 19. Best homogenization was achieved by the rotating knife method, better than by the use of a mortar or a shaker. Among the preferable standard commercial nutrients, "Oxoid Nutrient Agar", "Plate Count Agar" and the Hungarian product "Human Universal" are suitable for viable bacterial count estimation but culturing on "Oxoid Nutrient Agar" yielded higher counts. In routine work the less time-consuming and less laborious MPN method may be used for orientation. More exact estimations require one of the plate methods. On the basis of variance analysis they are equally suitable for total viable bacterial count estimation.

MICROBIOLOGICAL QUALIFICATION METHODS OF DRIED VEGETABLE PREPARATIONS

Ilona Fábri, Gy. Horváth, Erzsébet Murányi and E. Zukál

Central Institute for Food Control and Chemical Analysis, Budapest, County Institute for Food Control and Chemical Analysis, Kecskemét, Research Institute of Tinned Food and Paprika Industry, Budapest and Central Research Institute of Food Industry, Budapest

Dried vegetable preparations represent an important item of Hungarian food export. The international requirements are severe and well-defined. The fact that the control methods are different in the different countries, has caused difficulties in qualification. The germ count of coliform bacteria was estimated in three widely used media. The error originating in different factors was analysed by statistical-mathematical methods. The fiducial limits were calculated for the tests applied in the control of each type of dried preparation, taking into account the summarized error of the method.

EFFECT OF MECHANICAL SHAKING OF THE STOCK DILUTIONS ON THE MICROBIOLOGICAL QUALIFICATION OF FEEDS

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National Inspectorate for Feed Qualification and Control, Budapest

In the laboratory of the authors measured amounts of homogenized feed are diluted in 9 parts of sterile saline. This stock dilution is thoroughly shaken several times by hand so that the material is imbued by the diluent. In many other laboratories, the stock dilution is shaken mechanically. For comparative purposes, 16 samples of basic feed including cereals, sunflower seed, soy bean, peanut and meat meal, and 14 samples of mixed feed were examined for total germ count, mould number, bacterial and mould flora, using both technologies. Shaking was carried out in rubber-stoppered Erlenmeyer flasks in a shaking machine at 150 rpm for 5 or 10 minutes. Mechanical shaking of the stock dilutions for 5 or 10 minutes raised the bacterium count 1.7 to 48 times without causing any change in the composition of the bacterium flora. Under the same circumstances the conidium number increased 1.3 to 5000 times; the increase was especially marked if the original conidium number was low. Five and 10 min mechanical shaking raised the number of detectable mould species in 40 and 50%, respectively, of the samples. The high dilution to be employed in the case of a great rise in the conidium number may reduce the number of demonstrable species in the microflora, and thus lead to false conclusions. Mechanical shaking does not improve, in some respects even hinders, the microbiological qualification. Consequently, its introduction into routine feed control is not recommended.

MICROBIAL CONTAMINATION OF MACHINE-SLICED HAMS IN DIFFERENT SEASONS

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Microbial contamination of 87 ham samples has been determined before and after slicing in the Berkel apparatus. The analysis included the quantitative determination of the faecal indicator flora (coliform germs, *Enterobacteriaceae* negative for lactose hydrolysis and faecal streptococci) and the total germ count at 30°C under aerobic conditions. Every index showed a significant difference in favour of the winter season. The basic principles of hygienic ham slicing are summarized, specifying the tasks for the producers, for those selling ham, for the consumers and for the hygienic control service.

DEMONSTRATION OF THE ENTEROCOCCUS GROUP

J. Takács and S. Kovács

Central Laboratory of the Control Service of Meat Industry, Budapest

Food bacteriologists have made efforts to evolve a method suitable for the isolation of all members of the enterococcus group including bacteria relatively resistant to environmental effects. To find the most successful method, comparative studies have been made in well-known nutrient media, *viz.* 9 solid and 6 fluid media. Among the fluid media, the sodium-azide-glucose broth containing 0.003% bromthymol blue, among the solid media, Parker's agar proved to be the most suitable.

FOOD POISONING CAUSED BY STAPHYLOCOCCUS AUREUS

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In Hungary, food poisonings caused by *Staphylococcus aureus* have increased in number during the last decade. At present these are the most frequent food poisonings among those caused by known pathogens. This is due partly to the increasing diagnostic efficiency and partly to a true increase in number. Hygienic–epidemiological survey and clinical characterization of the cases are of great importance. Efforts should be made to isolate the pathogen(s) from the food as well as from secretions, and the hygienic error(s) allowing the microbe to multiply should be revealed. *S. aureus* strains qualified as pathogenic organisms occur in foodstuffs, as well as in stock and raw materials so frequently that their presence in itself does not make these materials objectionable. However, their high occurrence is a disturbing factor in clarifying their origin, the hygienic–epidemiological factors and their interconnections. To clarify these factors, the phage type and the antibiotic spectrum of the pathogen, the type of its enterotoxin, and, if possible, other factors such as the type of haemolysin and phosphatase and nuclease formation, should be established. Two kinds of deficiency are frequent in laboratory examinations: (i) the fine methods of identification are not applied, and (ii) only one strain is isolated, albeit one should reckon with the presence of different staphylococci in the same sample. In the evaluation of the results, especially in establishing the responsibility, it is most important to reveal the phase of processing in which an error could occur which may have led to microbial multiplication and toxin production.

SELECTIVE PROPAGATION OF CLOSTRIDIA IN MEDIA CONTAINING D-CYCLOSERINE TARTRATE

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It has been shown that clostridia except *Clostridium histolyticum* are not inhibited in their growth by 800 μg D-cycloserine per ml medium. *C. perfringens* strains are partially or completely inhibited by this concentration of D-cycloserine In semisolid media containing sulphite as reductant, and supplemented with egg emulsion to demonstrate lecithinase and lipase activity, D-cycloserine at 600 μg/ml proved satisfactory for the depression of the concomitant flora usual in food bacteriology, without disturbing the growth of any of the clostridium species. In plates of this medium neither sulphite reduction nor the lecithinase and lipase activities were disturbed. For selective propagation and differentiation of clostridia causing food poisoning the use of an agar medium based on the principle of sulphite reduction, supplemented with egg emulsion, is recommended. In such a medium the species *C. botulinum* and *C. perfringens* can be differentiated from other clostridia within 48 to 72 hours.

SIGNIFICANCE OF COLD-TOLERANT MICROBES IN THE DETERMINATION OF SEA FOOD PRODUCTS

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Cold-tolerant and psychrophilic microbes, not viable at 37°C, are of no importance in human medical microbiology. They may, however, deteriorate cold-preserved foodstuffs. The authors investigated the microflora of lobster samples which turned black while being boiled, using the methods prescribed for the detection of psychrophilic microbes. The samples were contaminated by the facultatively psychrophils *Proteus* or *Pseudomonas*. Blackening is attributed to a reaction product between some metabolites of the H_2S-producing *Proteus* and metal traces dissolved from the lobsters' crast. The source of contamination was supposedly the ice or the washing-water. Attention is called to the importance of cleanliness in processing foods of maritime origin.

MICROBIOLOGICAL EXAMINATION OF FEEDS

L. Etter

National Inspectorate for Feed Qualification and Control, Budapest

The following parameters are established for feeds: (i) total germ count (excluding moulds); (ii) aerobic microflora; (iii) coliform count; (iv) anaerobic spore-bearing (putrefying) count; (v) proteolytic bacterial count; (vi) mould count; (vii) demonstrability of salmonellae. These examinations are supplemented with mycotoxicological tests (aflatoxin, F-2). In the various kinds of feed about 70 species of bacteria have been encountered, most frequently micrococci, saprophytic and aerobic spore-bearing bacteria, and, mainly in cereals, flavobacteria. In a 12-month period, 439 samples of imported meat and fish meals were examined for salmonellae. From 35 samples (8%) a total of 15 *Salmonella* species were isolated. The total germ count did not exceed 500 000/g in 95% of the samples. Seventy-two species of fungi have been identified, of which 20 are toxin-producers. The most frequent 20 species belong to the genera *Aspergillus*, *Penicillium*, *Circinella*, *Mucor*, *Fusarium*, *Acremonium*, *Rhysopus*, *Hormodendrum*. Of these, 5 or 6 produce toxin. The mould count did not exceed 500/g in 64% of the samples. It exceeded 10 000/g in 4%. Aflatoxin B_1 was demonstrated in 175 (of these 158 were peanut samples) of the 243 feed samples tested in 1972. Of 285 samples, 38 contained F-2 toxin. Of the yearly 10 000 feed samples 75 to 80% proved microbiologically unobjectionable. The remainder was qualified as objectionable. This percentage is consistent with the occurrence in domestic animals of conditions attributable to feed.

A NEW MICROBIOLOGICAL METHOD FOR THE DETECTION AND QUANTITATIVE DETERMINATION OF T-2 FUSARIUM TOXIN

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An analytical microbiological method has been developed for the detection and quantitative determination of T-2 mycotoxin produced by *Fusarium tricinctum*. As test organism a *Prototheca* strain is used. For detection of the toxin the microbiological method is combined with thin-layer chromatography; in the case of quantitative determination a standard diffusion procedure is employed after preparative layer chromatography. The microbiological method is specific; its sensitivity is higher by one order of magnitude than that of the best chemical method.

INSTRUCTIONS TO AUTHORS

Manuscripts are accepted on the basis of scientific significance and suitability for publication on the understanding that they have not been published, submitted or accepted for publication elsewhere. Acceptance depends on the opinion of one or more referees and the decision of the Editorial Board. Each paper is dated the day the original manuscript was first received or the day the revision was received. Papers accepted for publication are subject to editorial revision.

Careful attention to the following points will aid rapid publication.

1. *General rules.* Emphasis is made upon simplicity of sentence structure and clarity of presentation. The manuscript should be typed double spaced on one side of the paper. Italicizing, except for headings of paragraphs, title of tables and for the usual scientific terms, is not permitted. Tables, figures, names, quotations and bibliography should carefully be checked for errors both in the manuscript and in the galley proofs.

2. *The first page* should be set out as follows.

(a) *A short title* of not more than 40 letters for use as a proposed running head.

(b) *The full title* should be one sentence containing preferably not more than 9 words and should clearly and precisely indicate the contents of the communication in adequate scientific terms.

(c) *Authors' names:* full first and family name for ladies, initials of first name(s) and full family name for gentlemen, *e.g.*

F. G. Kovács, Maria M. Szabó and G. Kádár

(d) *Authors' affiliations*, *e.g.*

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(e) *The Summary* should not exceed 180 English words. Dividing of the summary into paragraphs is not permitted. If the paper is published in a

language other than English, it must have an English title and English summary preceding the summary in the other language. The summary must clearly indicate the content, contain exact data for the important experimental results and should be informative rather than descriptive, *e. g.*: "Optimum conditions of continuous cultivation of *Bacillus* sp. strain 354 on collagen-derived substrate SP-100 are 36°C, pH 7.2 and dilution rate 0.25/hr" and not "The optimum conditions for the continuous cultivation of *Bacillus* have been investigated."

3. *The paper should be divided into the following parts:*

(a) *The introductory part* may contain a brief survey of the literature on the subject, with a description of the problem studied and the purpose of the work presented.

(b) *Materials and methods.* This part should be divided into paragraphs, *e.g. Bacterial strains. Culture media. Extraction of lipids. Statistical analysis.* Ingredients of media should be given as follows: Proteose peptone L46 (Oxoid, London), 10 g; monopotassium phosphate, 2 g; bromthymol blue 0.2% (w/v) aqueous solution, 12.5 ml; etc. Proprietary substances should, when first mentioned, be accompanied by the name and address of the manufacturer. A laboratory or chemical term not listed below may be abbreviated only after it has been written first in full with the abbreviation in brackets.

(c) *Results.* Description of the results should, when appropriate, be subdivided into parts with subheadings. Listing of data presented in the tables should be avoided in the text.

(d) *Discussion.* Repeating of the bibliography presented in the introduction ought to be avoided unless in order to compare literary data with the author's results. A recapitulation of the author's own results in the discussion is not permitted.

(e) *Acknowledgement.* Acknowledgements, including support by a grant, should be grouped into one paragraph.

(f) *References.* Only papers closely related to the work should be referred to. References should be cited by consecutive numbers in the text and so numbered and listed at the end of the paper without regard to alphabetical arrangement.

In the text references should be made by giving the author's surname, *e.g.* KRUEGER [1] or WESTPHAL and JANN [2]. When more than two authors are referred to, only the first author's name is given adding *et al.*, *e.g.* ALEXANDER *et al.* [3]. In the text bracketed figures indicating the corresponding references will also be sufficient instead of the authors' names, *e.g.* [4] or [5—9].

At the end of the paper the references should be listed as follows. Journals: names of all authors, journal (abbreviated according to the World Medical Periodicals List), volume, page number, year. Books: names of all authors, names of editors, title, volume, publisher, town, year, page number, *e.g.*

1. KRUEGER, W. W.: Principles of Microbiology. Saunders, Philadelphia—London 1953. p. 343.
2. WESTPHAL, O., JANN, K.: In WHISTLER, R. H. (ed.): Methods in Carbohydrate Chemistry. Vol. 5. Academic Press, New York 1965. p. 83.
3. ALEXANDER, A. D., LESSEL, E. F., EVANS, L. B., FRANCK, E., GREEN, S. S.: Int. J. Syst. Bacteriol. **22,** 165 (1972).
4. FELEKY, G., VASS, H. B.: Annual Meeting of the Hungarian Association of Microbiologists. Debrecen 1966.

(g) *Address of the author(s).* Full first and family names for both ladies and gentlemen and full mailing address should be given grouped according to the affiliation of the author(s), *e.g.*

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(h) *Footnotes.* Presentation of the paper at a meeting is cited in footnotes to the titles of articles. Other footnotes, except for tables, are not permitted.

4. *Tables.* All tables must be typed on separate sheets, must have descriptive headings and should be understandable without reference to the text. The tables should be numbered consecutively with Roman numerals.

5. *Figures.* Glossy photographic prints with strong contrast and/or graphs should be submitted with the manuscript. In the graphs the following point symbols should be used: ○ ● □ ■ △ ▲. The number of the figure, the author's name and, if necessary, an indication of the top of the illustration should be written on the back of each figure. The figures should be numbered consecutively with Arabic numerals and all legends typed in sequence on a separate sheet. Each legend should form one separate paragraph.

6. *Nomenclature of microorganisms.* Binomial names should conform to the International Rules of Nomenclature and to current terms of the International Committee on Systematic Bacteriology. Generic and specific name of the species should be written in full in the title, then where it first occurs in the summary and again in the text. In subsequent text the generic name should be abbreviated. The generic and generic-specific scientific name should be underlined for italics; a specific name should not be used without an accompanying capitalized generic name. Examples are as follows. *Staphylococcus*, *Shigella flexneri*, *Escherichia coli* serogroup O111 : K58 (in the following *E. coli* O111 : K58), *S. cholerae-suis* var. *kunzendorf*, *Bacillus* sp., *Sphaerophorus* spp., but ". . . a vehicle for shigella infection", ". . . the causative staphylococcus was present", ". . . salmonellae were detected". If it is thought advisable to mention the name(s) of the author(s) who first described the species or proposed the name of it, the name of the organism is written as "*Agrobacterium tumefaciens* (Smith and Townsend) Conn".

7. *Abbreviations.* Quantities should be expressed in Système Internationale (SI) units. Lengths should be given in nm (nanometres, 10^{-9} m) instead of millimicrons or in μm (micrometres, 10^{-6} m) instead of microns. Concentrations should be defined in terms of normality (N) or molarity (M); μg/ml, μg/g or μl/litre should be used instead of parts per million. The following abbreviations should be used:

AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-pyrophosphate
ATP	adenosine 5'triphosphate
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
A	ampere(s)
BCG	bacille Calmette Guérin
CM-cellulose	carboxymethylcellulose
CNS	central nervous system
CSF	cerebrospinal fluid
c. f. u.	colony-forming unit(s)
CF test	complement fixation test
cpm	counts per minute
cps	counts per second
Ci	curie(s)
c. p. e.	cytopathic effect
DNA	deoxyribonucleic acid
DEAE-cellulose	diethylaminoethylcellulose
e. o. p.	efficiency of plating
K	equilibrium constant
EDTA	ethylenediaminetetraacetate
GSSG	glutathione, oxidized
GSH	glutathione, reduced
g	gram(s)
g/100 ml	grams per cent
cal	gram calorie(s)
g	gravity
HAU	haemagglutinating unit
HI test	haemagglutination-inhibition test
Hz	Hertz (cycle per second)
hr	hour(s)
pH	hydrogen ion concentration, negative logarithm of
IU	international unit
i. d.	intradermal
i. m.	intramuscular
i. p.	intraperitoneal
i. v.	intravenous
INH	isoniazid
kg	kilogram(s)
km	kilometre(s)
LPS	lipopolysaccharide
log	logarithm
ln	logarithm, natural
litre(s)	litre(s)
($\times$600)	magnification
CPD_{50}	median cytopathogenic dose
ID_{50}	median infecting dose
LD_{50}	median lethal dose
ED_{50}	median protecting dose
$TCID_{50}$	median tissue culture infecting dose
m	metre(s)
Km	Michaelis constant
μCi	microcurie(s)
μEq	microequivalent(s)

μg	microgram(s)
μl	microlitre(s)
μm	micrometre(s)
μM	micromolar
μ mole(s)	micromole(s)
mA	milliampere(s)
mEq	milliequivalent(s)
mg	milligram(s)
mg/100 ml	milligrams per cent
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
mV	millivolt(s)
MIC	minimum inhibitory concentration
MLD	minimum lethal dose
min	minute(s)
M	molar
mole(s)	gram molecule(s)
mol wt	molecular weight
nm	nanometre(s)
nM	nanomolar
NAD^+	nicotinamide-adenine dinucleotide (oxidized form)
NAHD	nicotinamide-adenine dinucleotide (reduced form)
$NADP^+$	nicotinamide-adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
N	normal
No.	number(s)
5×10^3	number of bacteria, etc.
OD	optical density
α	optical rotation
PAS	para-aminosalicylic acid
%	(with numeral) per cent
pM	picomolar
p. f. u.	plaque-forming unit(s)
P	probability
^{131}I, [^{14}C] glucose	radioactivity
RBC	red blood cell (count)
®	registered trademark
R_F	retardation factor
rpm	revolutions per minute
RNA	ribonucleic acid
s	second(s) (time)
s	sedimentation coefficient
sp.	(with generic name) species, singular
spp.	(with generic name) species, plural
SA	specific activity
$[\alpha] \frac{t}{\lambda}$	specific rotation
cm^2	square centimetre
SD	standard deviation
SEM	standard error of the mean
s. c.	subcutaneous
S	Svedberg unit of sedimentation coefficient (10^{-13} s)
t test	"t" test
torr	unit of pressure (mm Hg)
UV	ultraviolet
U	unit(s)
VA	voltampere(s)
vol	volume(s)

(v/v)	volume in volume
W	watt(s)
λ	wavelength
wt	weight
(w/v)	weight in volume
(w/w)	weight in weight
WBC	white blood cell (count)

8. *Proofs.* Two consecutive sets of proofs are provided before publication. Alterations on the proof not conforming to the text of the original manuscript should be avoided. Proofs are to be returned within 48 hours of receipt (by air mail where appropriate) to the Editor.

9. *Reprints.* One hundred and fifty copies of each article are granted free of charge. Requests for additional quantities should be sent to the Editor.

The Editor

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FELHÍVÁS A SZERZŐKHÖZ

Folyóiratunk a dolgozatokat tudományos értékük és közlésre való alkalmasságuk szerint fogadja el, azzal a feltétellel, hogy azokat világnyelveken más lapokban még nem közölték, ill. közlésük nincs folyamatban. A dolgozatok elfogadásáról egy vagy több szakbíráló véleménye alapján a Szerkesztő Bizottság dönt. A közlemények az érkezés napjának keltezésével, ill. — ha javításuk hosszabb időt vett igénybe — a helyesbített példány érkezésének keltezésével jelennek meg. A közlésre elfogadott dolgozatokban a szerkesztőség nyelvi, formai és terjedelmi szempontból javításokat eszközölhet.

A gyors megjelenés érdekében kérjük, tartsa be a következőket:

1. *Általános szabályok.* A dolgozat mondatszerkezete egyszerű, fogalmazása világos legyen. A kéziratot kettős sorközzel, a papír egyik oldalára gépeljük. Dőlt betűkkel csak a bekezdések és táblázatok címe és a szokásos tudományos kifejezések szedhetők. A táblázatok, ábrák, nevek, idézetek és az irodalom ellenőrzése a szerző kötelessége, mind a kéziratban, mind a kefelevonatokban.

2. *Az első oldal* szerkezete a következő legyen:

(a) Nem több, mint 50 betűből álló, rövid cím, mint ajánlott élőfej (magyarul beadott dolgozatoknál elhagyható).

(b) A *teljes cím* egy mondat legyen, mely szakkifejezésekkel, világosan mutatja a közlemény tartalmát. Legmegfelelőbb, ha nem több, mint 9 idegen szóval kifejezhető.

(c) A *szerzők neve* (női szerzők teljes kereszt- és vezetékneve, férfi szerzők keresztnevének kezdőbetűje és teljes vezetékneve). A keresztnevek írhatók magyarosan vagy idegen nyelven, lényeges, hogy a szerző azt a nevet használja, amely alatt eddigi közleményei megjelentek. Pl.

F. G. Kovács, Maria M. Szabó and G. Kádár

(d) Az az intézet, ahol a szerzők a munkát végezték (nem szükségszerűen azonos a szerzők jelenlegi címével, melyet a dolgozat végén kell feltüntetni), pl.

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Magyar nyelven beadott dolgozatban is az intézet hivatalos idegen nevét kell használni, ezt ugyanis a fordító nem mindig ismeri.

(e) Az *összefoglalás* nem haladhatja meg a 180 angol szót, nem osztható fel bekezdésekre. Ha a dolgozatot nem angolul közlik, a kérdéses nyelven írt összefoglalást meg kell előznie egy angol nyelvű címnek és összefoglalásnak. Az összefoglalás világosan fejezze ki a tartalmat, pontos adatokkal, pl. „A *Bacillus* sp. 354 jelzésű törzs folyamatos tenyésztésére kollagen-eredetű SP-100 tartalmú táptalajban a legalkalmasabb körülmények a következők: 36°C, pH 7,2, hígítási arány 0,25/óra." Ne írjuk így: „Egy *Bacillus* törzs tenyésztésének legalkalmasabb körülményeit vizsgáltuk."

3. A *szöveg* a következő részekre osztható:

(a) A *bevezető rész* tartalmazhatja a tárgyhoz tartozó irodalom rövid összefoglalását, a vizsgált kérdés leírását és a munka célkitűzését.

(b) *Anyagok és módszerek.* Ezt a részt bekezdésekre kell bontani, pl. *Baktériumtörzsek. Táptalajok. Lipidkivonás. Statisztikai elemzés.* Táptalajok alkatrészeit a következőképpen adjuk meg: Proteose pepton L46 (Oxoid, London), 10 g; káliumdihidrofoszfát, 2 g; brómtimolkék, 0.2% (w/v) vizes oldata, 12,5 ml; stb. Védett nevű anyagok nevének első alkalommal való használatánál zárójelben fel kell tüntetni a gyártó cég nevét és telephelyét. Laboratóriumi vagy kémiai kifejezések — az utasítás angol változatában felsoroltak kivételével — csak akkor rövidíthetők, ha előzőleg teljes nevüket kiírtuk és a rövidítést utána zárójelben közöltük.

(c) *Eredmények.* Az eredmények leírását, ha lehetséges, alcímekkel ellátott bekezdésekre kell bontani. A táblázatokban bemutatott adatok felsorolását a szövegben mellőzni kell.

(d) *Megbeszélés.* A bevezető részben már említett irodalom nem ismételhető a megbeszélésben, kivéve, ha az adatokat a szerző saját eredményeivel kell összehasonlítani. A dolgozat tárgyát képező eredményekre vonatkozó adatok, melyeket a szerző az „Eredmények" részben már leírt, nem ismételhetők meg összefoglalásszerűen a megbeszélésben.

(e) *Köszönetnyilvánítás.* Egyetlen bekezdés legyen.

(f) *Irodalom.* Csak a munkával szorosan összefüggő irodalom idézhető. Az irodalmat a szövegben folyamatos számozással kell ellátni és ugyanilyen sorrendben (tehát nem abc szerint) kell a kézirat végén felsorolni.

A szövegben az irodalmat a szerző vezetékneve szerint kell említeni, pl. KRUEGER [1], vagy WESTPHAL és JANN [2]. Ha több, mint két szerzőt idézünk, az első szerző nevét kiírjuk és utána az „*és mtsai.*" jelzést alkalmazzuk, pl. ALEXANDER *és mtsai* [3]. A szövegben az is elegendő, ha a szerző neve helyett csak a megfelelő sorszámot (sorszámokat) írjuk, pl. [4], vagy [5—9]. A dolgozat végén az irodalmat a következőképpen kell felsorolni. Folyóiratok: az összes szerzők neve, a folyóirat (nemzetközi rövidítéssel), kötet, kezdő oldalszám, év. Könyvek: az összes szerzők neve, a szerkesztők neve, cím, kötet, kiadó, város, év, oldalszám. Pl.

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(g) A *szerzők címe*. Mind női, mind férfi szerzőknél a teljes nevet és a teljes címet kell feltüntetni, a szerzők munkahelyének megfelelő csoportosításban, pl.

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(h) *Lábjegyzetek*. Ha a közlemény már előadásban elhangzott, a kérdéses rendezvény pontos meghatározásával a címhez tartozó lábjegyzetben közölhető. Más lábjegyzetek (a táblázatok kivételével) nem használhatók.

4. *Táblázatok*. Minden táblázatot külön oldalra kell gépelni, a tartalmat kifejező címmel. A táblázat a szöveg olvasása nélkül is érthető legyen. A táblázatokat római számmal kell jelölni.

5. *Ábrák*. A fényes, kontrasztos fényképek, ill. az ábrák a kézirathoz mellékelendők. Az ábrákban a következő pont-jelzések használhatók: ○ ● □ ■ △ ▲. Az ábrák sorszáma, a szerzők neve és, ha szükséges, felfelé irányuló részük a hátoldalon jelölendő. Az ábrákat folyamatosan arab számokkal jelöljük és szövegüket külön lapon, sorrendben gépeljük. Az ábra szövege egy bekezdést alkosson.

6. *Mikroorganizmusok neve*. Az elnevezések feleljenek meg a nemzetközi nómenklatúra szabályainak és Nemzetközi Taxonómiai Bizottság legújabb határozatainak. A faj generikus és specifikus nevét teljesen ki kell írni a címben, majd az összefoglalóban, ill. a szövegben, amikor először említjük azokat. A továbbiakban a generikus nevet rövidítjük. A generikus, ill. generikus-specifikus nevet alá kell húzni, hogy azokat dőlt betűvel szedjék; a specifikus név az előtte levő nagy kezdőbetűjű generikus név nélkül nem használható. Pl. *Staphylococcus*, *Shigella flexneri*, *Escherichia coli* szerocsoport O111 : K58 (a továbbiakban *E. coli* O111 : K58), *S. cholerae-suis* var. *kunzendorf*, *Bacillus* sp., *Sphaerophorus* spp., de: „a shigella fertőzés terjesztője", „a kórokozó staphylococcus", „salmonellákat mutattak ki." Ha azoknak a szerzőknek a neveit meg kívánjuk említeni, akik a speciest először írták le, ill. először adtak nevet neki, a mikroorganizmus nevét a következőképpen írjuk: „*Agrobacterium tumefaciens* (Smith and Townsend) Conn".

7. A mennyiségeket a „Système Internationale" (SI) egységek szerint kell kifejezni. A hosszúságokat nm (nanométer, 10^{-9} m), ill. μm (mikrométer, 10^{-6} m) jelölje millimikron, ill. mikron helyett. Koncentráció jelzésére N (normál), ill. M (moláris) jelzést alkalmazzunk. A „parts per million" helyett μg/ml, μg/g, vagy μl/liter jelölést használjunk. Az alkalmazandó rövidítéseket az angol változatban tüntettük fel.

8. *Kefelevonatok.* A szerző két egymás utáni kefelevonatot kap. Olyan javítások, melyek nem felelnek meg a kézirat szövegének, nem eszközölhetők. A kefelevonatokat 48 órán belül vissza kell küldeni a Szerkesztőségnek.

9. *Különlenyomatok.* 150 különlenyomat térítésmentes. További különlenyomatokat a Szerkesztőségtől kell igényelni.

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DIFFERENTIATION OF BACTERIA BY INDOXYL BUTYRATE PAPER STRIP METHOD

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(Received July 13, 1972)

Summary. A paper strip method for indoxyl butyrate hydrolysis has been worked out by which the esterase activity of bacteria can rapidly and easily be detected. The procedure has proved satisfactory for determining the number of esterase producing colonies on agar streak plates, and it is believed to find its place also in identification of bacteria.

In determining the characteristics of bacteria the detection of esterases is often necessary. Among these, lipase and phosphatases are the ones usually studied. In this paper, detection of indoxyl butyrate hydrolysis is discussed.

Several materials are suitable for the demonstration of bacterial ester hydrolysis. Fats and oils are less applicable and therefore rarely used unless the aim is to demonstrate the ability of bacteria to hydrolyze them. It is more practical to use esters which are easily emulsified and evenly incorporated in media and give more reliable results. Besides fats [1—3], tallow [4] and oil, a number of substances have been employed, such as tributyrin [5—7], Tweens, sorbitane esters [8—11], indoxyl acetate and indoxyl butyrate [12].

Using our new paper strip method for indoxyl butyrate hydrolysis we have performed comparative studies on the breakdown of indoxyl butyrate, fats, stearin, tributyrin and Tweens. Experiments were also done with lecithinase production and the cleavage of acetylcholine bromide. It was found that indoxyl butyrate is suitable as a substrate and is a sensitive index of ester hydrolysis.

Materials and methods

Indoxyl butyrate hydrolysis. An 0.25% solution in ethyl alcohol was made from indoxyl butyrate (L. Light Co., England). Filter paper (preferably Schleicher–Schüll's No. 2043/b) was soaked in this solution and dried at room temperature. The sheets of filter paper were cut in strips of adequate size (*e.g.* 0.5 × 1.0 cm). Dry strips in closed bottles can be stored in dark for long periods. Best results are obtained with 24 hr cultures on nutrient agar, but any other medium can be applied, provided that its colour does not interfere with that indicating a positive reaction. Indoxyl butyrate strips are laid directly on surface colonies. Indoxyl butyrate hydrolysis is indicated by the deep blue colour of indican, developing soon after the strip has become wet. A prompt change of colour (usually within 2 min) is marked with ++, while + means a less intensive blue colour or delayed appearance (but in less than 10 min).

Distinct spots appear if there are isolated colonies on the plate, and an even bluish colour develops in contact with confluent dense growth.

The number of esterase positive colonies in mixed surface cultures can also be determined by means of the indoxyl butyrate strips, on which they are represented by blue points.

Alternatively, the test can be carried out by rubbing a loopful of the growth from a pure culture on the indoxyl butyrate paper strip, as with the performance of the oxidase test.

Table I

Results of comparative experiments

Organism	No. of strains	Indoxyl butyrate		Tweens		Acetylcholine bromide	
		+	%	+	%	+	%
Staphylococcus spp.	156	155	99.3	133	85.2	45	28.8
Bacillus spp.	201	162	80.5	104	51.7	53	26.3
Pseudomonas aeruginosa	40	40	100.0	24	60.0	8	20.0
Total	397	357	89.9	261	65.7	106	26.7

Hydrolysis of fat. To peptone water containing phenol red, 2% pork fat is added. After being adjusted to pH 7.0 and autoclaved the medium is ready for use. Breakdown of fat is indicated by a change of colour from red to yellow. Reading is performed at 24 and 48 hr.

Hydrolysis of tallow was examined by the method of LOVREKOVICH [4]. A paper strip impregnated with tallow is immersed in the broth culture of the organism to be examined. Staining of the paper strip with methylene blue is taken as the evidence of hydrolysis.

Hydrolysis of tributyrin. The nutrient base is peptone water containing phenol red, to which tributyrin (glycerol tributyrate, B.D.H.) is added to give a final concentration of 1.5%. The butyric acid resulting from the hydrolysis of tributyrin acidifies the medium and the colour of the indicator turns yellow. Reading is performed after 24 and 48 hr of incubation.

Hydrolysis of Tweens (sorbitane esters). The method of SIERRA [13] was adopted with the modification that the concentration of the substrate was 0.5%. Hydrolysis is indicated by precipitate formation. Reading is done after 24 and 48 hr.

Hydrolysis of lecithin. To 200 ml melted agar base at about 50°C, the yolk of one egg was mixed. Hydrolysis of lecithin was apparent from the opacity around the colonies.

Hydrolysis of acetylcholine bromide. The medium was prepared by adding acetylcholine bromide to peptone water containing phenol red. By the hydrolysis of the substrate acetic acid is formed, which acidifies the medium.

Results

Hydrolysis of the above seven substrates was investigated with bacteria that were expected to give positive reaction. One hundred and fifty-six strains of *Staphylococcus aureus*, 40 of *Pseudomonas aeruginosa* and 201 of different aerobic sporebearers were studied. Most positive reactions were observed with indoxyl butyrate paper strips, less were obtained with the Tweens and in decreasing order with tallow, acetylcholine bromide, tributyrin, fat and lecithin. Results are summarized in Table I.

Naturally, divergent results were obtained with different substrates. Positivity depended on the strain under study, the nature of the substance and the sensitivity of the test. The results obtained with indoxyl butyrate and the Tweens corresponded most closely.

After this comparative series of experiments we performed more extensive studies by applying our new indoxyl butyrate method. Results obtained with different organisms are presented in Table II.

with different substrates

Tallow		Tributyrin		Fat		Lecithin	
+	%	+	%	+	%	+	%
54	34.6	31	19.8	22	14.1	19	12.1
44	21.8	52	25.8	34	16.9	37	18.4
28	70.0	8	20.0	7	17.5	—	0.0
126	31.7	91	22.9	63	15.8	56	14.1

Table II

Hydrolysis of indoxyl butyrate by different organisms as tested by the paper strip method

Organisms	No. of strains tested	No. of strains giving positive reaction		No. of strains giving negative reaction	Percentage of	
		++	+		positives	negatives
Escherichia coli	403	—	72	331	17.8	82.2
Klebsiella, Enterobacter	105	—	30	75	28.5	71.5
Proteus	137	—	—	137	—	100.0
Pseudomonas aeruginosa	71	71	—	—	100.0	—
Staphylococcus aureus	124	124	—	—	100.0	—
Staphylococcus epidermidis	50	43	—	7	86.0	14.0
A and D group streptococci	257	—	—	257	—	100.0
Diplococcus pneumoniae	46	—	—	46	—	100.0
Bacillus spp.	68	57	—	11	83.0	17.0

++ = intensive blue colour appearing within 2 min
+ = less intensive blue colour appearing in less than 10 min

In some cases positivity was shown by organisms that had not been expected to behave so. The *Enterobacteriaceae* were studied most extensively as we wished to see the regularity of positive reactions and to know whether the indoxyl butyrate paper strip method could be utilized for their differentiation or for the verification of their diagnosis.

Results for esterase activity are shown in Table III.

Some organisms yielded always positive or negative reaction. Further, *Escherichia coli* and *Yersinia enterocolitica* strains of animal origin were consequently positive. We believe, therefore, that this observation, if it will be

Table III

Esterase activity

Organisms	No. of strains	++	+	–
Arizona	30	—	27	3
Bacillus spp.	21	3	5	13
Citrobacter	30	1	8	21
Escherichia coli associated with infantile enteritis	23	11	10	2
Eschrichia coli of animal origin	14	—	14	—
Morganella	30	—	—	30
Pasteurella multocida	3	—	—	3
Proteus mirabilis	29	2	1	26
Proteus vulgaris	29	—	—	29
Providencia	30	4	8	18
Rettgerella	30	—	1	29
Salmonella	50	—	23	27
Shigella boydii group	32	—	6	26
Shigella dysenteriae group	27	—	2	25
Shigella flexneri	26	3	19	4
Shigella sonnei	5	—	—	5
Yersinia enterocolitica of human origin	24	—	—	24
Yersinia enterocolitica of other origin	10	—	10	—
Yersinia pseudotuberculosis	25	—	10	15

++ = intensive blue colour appearing within 2 minutes
+ = less intensive blue colour appearing in less than 10 minutes
— = negative

confirmed by the investigation of an appropriate number of strains, may provide a means of distinguishing these from strains of human origin.

Discussion

A comparison of the results obtained with indoxyl butyrate strips with those shown by other substrates was made in search for a reliable test for bacterial esterase. The action of bacterial enzymes on different substrates was not uniform and the sensitivity of the substances was also different. The esterase tests employing different substrates cannot substitute each other. If the production and the action of the esterase is to be detected, the indoxyl butyrate paper strip method should be the indicator of choice, since of all

the methods it is the one most sensitive, rapid, inexpensive, easy to prepare and allows a good colour differentiation. A rapid hydrolysis of the substrate is produced by the enzyme, during which the indoxyl compound formed is oxidized to indican, the latter being deep blue in colour. The chemical explanation of this process has been given by GIBBS and SKINNER [12].

Thus, our experience indicates that the indoxyl butyrate paper strip method as a biochemical test may find its place in strain identification and verification.

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EFFECT OF SOME FACTORS ON THE BIOLOGICAL ACTIVITY OF MEASLES VIRUS

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Summary. The detectability of morbilli virus strain L-16 in monkey kidney cell line III/1 was not influenced either by the age of the cells, the number of cells per tube, the amount of inoculum (neg. log $TCID_{50}$ 2.30—2.72 in 0.1—1 ml) or by the serum content of the maintenance medium. An unfavourable effect on the detectability of the virus inoculated was observed if Hanks' solution alone or Parker's synthetic medium 199 supplemented with bovine amniotic fluid was used for maintenance or if the virus inoculated was left to adsorb to the cells for 10–60 minutes before flooding with maintenance medium. Optimum results were obtained when Parker's synthetic medium 199 alone was used as maintenance medium and when the cultures inoculated were incubated at 34°C.

Factors influencing the determination of the biological activity of measles virus in cell cultures have recently been studied by several investigators [1—3]. The problem is of practical significance in the quantitative evaluation of live measles vaccines.

The purpose of the present studies has been to determine whether the propagation of measles virus may be influenced by the following factors: (i) the age and the number of cells in the cell cultures used; (ii) the amount of inoculum; (iii) the composition of the medium; (iv) incubation temperature; and (v) duration of virus adsorption.

Materials and methods

Virus. The attenuated strain Leningrad-16 (L-16) of measles virus was used.

Cell culture. Rhesus monkey kidney cell line III/1 was used [4–6]. The propagation medium consisted of 45% Hanks' BSS, 40% Parker's synthetic medium 199, 10% bull serum, and 5% lactalbumin hydrolysate solution. In general, a cell number of 1×10^5, but for studying the effects of varying number of cells, cell numbers of 2×10^5 and 4×10^5 were also used. The maintenance medium contained 2% bull serum in Parker's synthetic medium 199. Other compositions of the maintenance medium are indicated in the text and in the Tables. Cell cultures were infected 3–4 or 3, 6, 8, and 10 days after trypsinization.

Titration of virus. Virus titrations were performed according to SHIKINA *et al.* [7]. In order to determine the quantity of virus used for infection, a tube culture was inoculated with 0.1, 0.3, 0.5, 0.8, and 1 ml of the virus. The contents of 3–5 ampoules containing virus L-16 (2.5 ml freeze-dried virus suspension/ampoule) were pooled for every titration. At each dilution of the virus, 4–10 tubes were infected and incubated at 34°C and at 32, 34, 37, and 40°C. $TCID_{50}$ values were determined according to the method of Reed and Muench on the 14th day of incubation.

For studying the adsorption of measles virus, the viruses inoculated into cell cultures were let to adsorb at room temperature for 10, 30, and 60 minutes. The part of virus failing to adsorb was then washed off the cell surfaces by three changes of medium. Finally, the cultures were flooded with the maintenance medium consisting of 2% bull serum and 98% Parker's medium 199.

Results

The monkey kidney cell line III/1 has been used for the control of measles vaccine since about 1966. Virus infection resulted in well observable cytopathic effects. They consisted of the formation of syncitia, multinucleated giant cells, and vacuoles increasing in size and number parallel to the process of virus propagation. Nuclear inclusions characteristic of measles virus could not be detected after haematoxylin–eosin staining.

Cell number and the age of cell cultures. III/1 cells produced completely confluent monolayers 3 days after starting the cultures when a cell number of 10^5 cells/tube was used. Cell density showed a slight increase by the 6th, 9th, and 10th days of incubation but the morphology of the cultures remained essentially unchanged except for a sporadic occurrence of a few rounded cells observable in the cell layer and some detached cells present in the medium. As shown in Fig. 1, neither the age nor the number of the cells had an influence on the quantitative detectability of the inoculated virus.

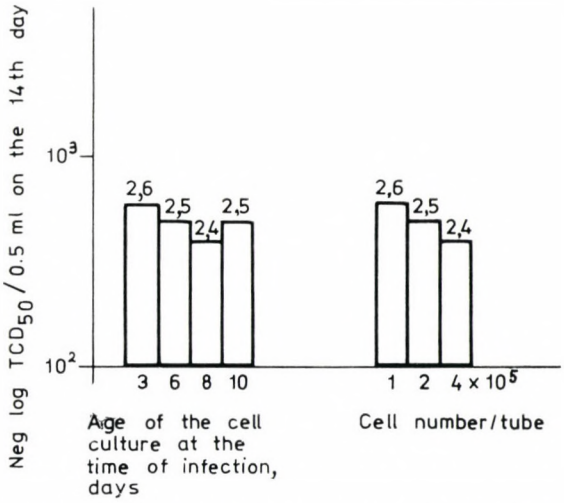

Fig. 1. Effect of age and of number of cells per tube on the detectability of virus L-16 in cell line III/1

Amount of inoculum. Using inocula of 0.1, 0.3, 0.5, 0.8, and 1.0 ml, virus titres calculated for 0.5 ml showed a fluctuation within 0.4 log (Table I). The range of fluctuation was independent of the amount of virus inoculated and it was within the limits of "biological" deviation. The inoculated amount of virus had no significant influence on its detectability.

Composition of the maintenance medium. The effects on the detectability of the inoculated virus of different concentrations of calf and bull sera (2 and 5%), bovine amniotic fluid (BAF) and human albumin were studied. The basic

Table I

Effect of the amount of inoculum on the quantitative detectability of the inoculated measles virus strain L-16

Inoculum, ml	Neg log $TCID_{50}$/0.5 ml inoculum
0.1	2.31
0.3	2.72
0.5	2.32
0.8	2.63
1.0	2.30

Table II

Effect of the serum content of maintenance medium on the quantitative detectability of inoculated measles virus

Maintenance medium	Serum concentration, per cent	Neg log $TCID_{50}$/0.5 ml on 14th day
199 + normal	2	2.61
bull serum	5	2.79
199 + normal	2	2.63
calf serum	5	2.64
199	0 without serum	2.71

solution was supplemented with the components shown in Tables II and III The detectability of the virus inoculated was not influenced by either the presence (2 and 5%) or the absence of calf and bull sera (Table II). The same held for the absence and presence in a concentration of 2% of rabbit and rooster sera and human albumin (Table III). On the contrary, BAF inhibited the detectability of the virus: the titre recovered was 0.6—0.9 exponent lower than when maintenance media containing no serum or human albumin or serum were used. Hanks' BSS used unsupplemented also exerted an unfavourable effect on the detectability of the virus.

Incubation temperature of infected cell cultures. The tube cultures infected were incubated at 32, 34, 37 or 40°C. The titres obtained at various incubation temperatures are shown in Fig. 2. At 40°C, no cytopathic effect developed even if infection was made with undiluted virus (10°), indicating that virus propagation was completely inhibited at this temperature. The optimum temperature for the titration of L-16 virus was 34°C.

Duration of adsorption. Using the method of SHIKINA *et al.* [7], control

Table III

Effect of the composition of maintenance medium on the quantitative detectability of inoculated measles virus strain L-16

Composition of maintenance medium	Neg log $TCID_{50}$/0.5 ml at the 14th day
199 + 2% rabbit serum	2.28
199 + 2% rooster serum	2.16
199 + 2% human albumin	2.56
199 + 50% BAF	1.62
199	2.25
Hanks	1.71

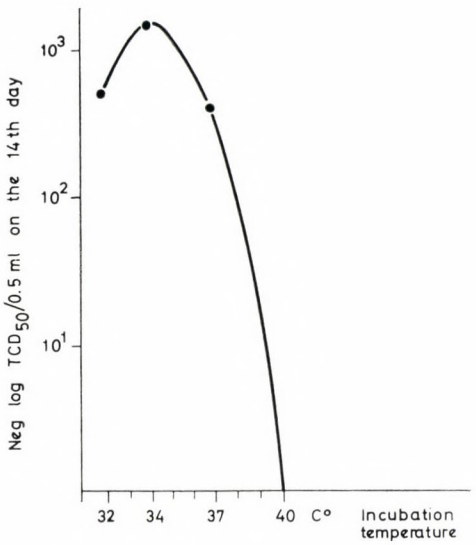

Fig. 2. Effect on the detectability of inoculated virus of incubation temperature of infected cell cultures

tubes were prepared by adding different dilutions of the virus (in 0.5 ml) to 0.5 ml aliquots of Parker's medium 199 without preliminary adsorption. The tube cultures thus infected were placed into an incubator. A preceding adsorption of the same amounts of virus for 10, 30, and 60 min exerted an adverse effect on the detectability of the inoculated virus as compared to the controls (Fig. 3).

Discussion

There are several factors influencing the sensitivity of cell cultures to viruses. The effects of the age of cell cultures [8, 9, 10] and the composition

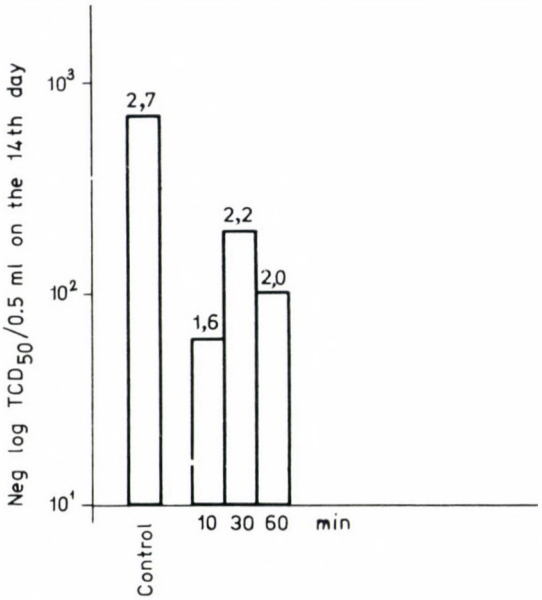

Fig. 3. Effect on the detectability of the virus of adsorption time (10, 30 and 60 min, control 0 min) of virus inoculum

of the medium [11, 12] on the detectability of adeno- and polioviruses [13] have been studied. In our knowledge, however, there is no report on the factors influencing the detectability of measles virus strain L-16. Studying the ESCs strain of measles virus in vervet monkey kidney cells of different ages, POPOVA [3] found that the sensitivity of cells with regard to the detectability of the virus decreased with the age of the cultures (up to the 7th—15th day) and optimum results could be obtained when 9-day-old cultures ($1.5—3.0 \times 10^6$ cells/flask) were used. Our results showed that neither the age (within 10 days) nor the number of cells per tube (10^5, 2×10^5, and 4×10^5) influenced the detectability of virus L-16 in cell line III/1. As to the amount of inoculum used for the infection of tube cultures, POPOVA [3] concluded that the adsorbed amount of virus was inversely proportional to the amount of inoculum. Consequently, it was useless to increase the amount of inoculum since virus titres obtained after an optimum threshold value showed a decrease rather than an increase. Under our experimental conditions, when other disturbing factors (*e.g.* the effect of light) were excluded, no correlation between the amount of inoculum and the titres extrapolated to 0.5 ml virus inoculated in volumes ranging from 0.1 to 1.0 ml, was found.

The role of the serum content of the medium in the propagation of measles virus was pointed out by findings [14] suggesting that the amount of serum necessary to obtain confluent cell cultures was amply sufficient for

the propagation of measles virus. Supporting this, the results of our own studies showed that even Parker's medium 199 alone was sufficient for the propagation of measles virus strain L-16 insofar as the cell culture itself could be maintained. The addition of serum independent of its origin or concentration applied, failed to influence the results. On the other hand, BAF at a concentration of 50% inhibited the detectability of virus (leading to a decrease of log 0.6 virus in yield), while Hanks' BSS used unsupplemented as maintenance medium was insufficient even for a 14-day survival of the cells (spontaneous degeneration of the cells occurred). MATUMOTO [1] found incubation temperatures of 32.2 and 35.5°C more adequate than 37.0°C for the propagation of measles virus. SHIKINA *et al.* [7] reported that the optimum incubation temperature was 35—36°C for strain L-16. Our own results were consistent with the above data. Previous adsorption to the cells of measles virus strain L-16 was shown by our results to have had an adverse rather than a favourable effect on the detectability of virus, and titration by the method of SHIKINA *et al.* [7] was found most adequate. The tube cultures infected were carefully protected from light during the period of virus adsorption: it was only the time taken by the addition of maintenance medium after adsorption that the infected tubes were exposed to the effect of light. This, however, may have accounted for the differences observed in the titre values. Besides, an adsorption time of 10 minutes was too short as shown by the 0.6 log increase in titre obtained after 30 min adsorption of the virus.

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AUSTRALIA ANTIGEN SURVEY IN HUNGARY

III. MORPHOLOGICAL STUDY OF THE ANTIGEN BEFORE AND AFTER TREATMENT WITH TWEEN 80

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Summary. (i) Twoo pools of Australia (Au) antigen-positive blood samples were centrifuged at 46 000 *g* for two hours. The sediments were resuspended in 1/20th volume of PBS and after a clarifying centrifugation one half of the material was treated with 0.5% Tween 80, the other half served as control. A second centrifugation was carried out at 23 000 *g*. Complement-fixing (CF) antigen activity of the fractions was measured using a serum sample obtained from a patient with haemophilia A. (ii) After the centrifugation at 46 000 *g* 1/60th of the original CF activity was recovered in the sediment. This sediment was relatively rich in Dane particles 40 to 45 nm in diameter and somewhat larger (45 to 60 nm) particles of similar virion-like structure. Spherical and filamentous structures 20 nm in diameter were also present. The same kinds of particles were found in ultra-thin sections. Here the particles 40 to 60 nm in diameter showed a double outer coat and an electron-dense inner core. Besides, still larger, non-virion-like, approximately empty structures limited by a simple membrane were seen. (iii) Tween 80 treatment injured the outer coat, and revealed the inner structures of Dane particles. The majority of the larger particles appeared as empty flattened vesicles, the remainder retained some of the inner structures. The loss of CF activity was negligible during Tween 80 treatment and subsequent centrifugation. (iv) Both the intact particles and the released inner structures were involved in the immuno-electronmicroscopic reaction performed with the antibodies in the anti-Au positive haemophiliac serum, suggesting that a great variety of particles possessed some specificity related to the hepatitis B antigen.

Bayer *et al.* [1] were the first to demonstrate spherical and filamentous particles 19 to 21 nm in diameter in serum samples from subjects with Australia (Au) antigenaemia. In some of the particles a well-defined substructure, *viz.*, a central core, was recognized. These authors as well as Almeida *et al.* [2] reported on the electron-microscopic immunoprecipitation of these particles. The spherical and the filamentous forms were of the same immunological specificity [3]. Ether treatment led to complete destruction of the filamentous forms while the spherical forms, losing their outer coat, retained their particulate character [4, 5]. Dane *et al.* [6] described a third type of particle of Au antigen specificity. These are spherical forms 42 nm in diameter, showing a well-defined substructure consisting of an inner core surrounded by a shell and an outer coat. Dane *et al.* supposed that these particles, now called Dane particles, are infectious virions, whereas the other forms mainly consist of coat material. Zuckerman *et al.* [7, 8] on the other hand are of the opinion that the Dane particles are coiled up aberrated filaments, and only some of the small forms might be infectious.

ALMEIDA *et al.* [9], having modified the method described by GERIN *et al.* [10], treated preparations mainly consisting of Dane particles with Tween 80. Immuno-electron microscopy then revealed that the core of the Dane particles which was made accessible for antibodies by the Tween 80 treatment, was different in antigenicity from the outer coat; against the core antigen patients with acute hepatitis developed antibodies only during convalescence. Polytransfused haemophiliacs possess both anti-core and anti-Au antibodies.

In our own work the electron-microscopic investigations of ALMEIDA *et al.* [3] have been reproduced in a slightly modified form. The modification had to be introduced to make the Tween-80-treated preparation suitable for using it as antigen in microcomplement-fixation (CF) tests which, unlike immuno-electron microscopy, can be applied in mass investigations.

Materials and methods

Preparation of Au antigen. Donor bloods having proved positive for Au antigen [11] were pooled. Each of the two pools used in the present experiments was 3 litres in volume, having been collected from 14 and 18 donors, respectively. The pools were stored in a deep-freezer, thawed, then centrifuged for 30 min at 2000 rpm. Samples from both supernatants and the resuspended sediments were stored in the frozen state. The bulk of the supernatants was then centrifuged at 15 000 rpm, nominally representing 46 000 *g*, for 2 hr at 4°C, using the 6×500 ml angle rotor of the Janetzki Vac-20 centrifuge. The sediment was resuspended in 150 ml PBS by intensive pipetting and subjected to clarifying centrifugation for 10 min at 2000 rpm.

Tween-80 treatment. The above supernatant was halved: to the one half 0.5% Tween 80 was added, the other half served as control. Both were allowed to stand at room temperature for 30 min, then centrifuged for 2 hr at 23 000 *g* in the Janetzki T24 centrifuge. The sediments were resuspended in 75 ml barbiturate-buffered saline. After another clarifying centrifugation the supernatants were used as antigen.

Barbiturate-buffered saline. Solution No. 1 contained 4.6 g diethylbarbituric acid dissolved in 500 ml distilled water. Solution No. 2: NaCl, 83.8 g; $NaHCO_3$, 2.52 g; sodium diethylbarbiturate, 3.0 g; $MgCl_2 \cdot 6H_2O$, 10 g; $CaCl_2 \cdot 2H_2O$, 0.2 g, dissolved in 1500 ml distilled water. The two solutions were mixed and autoclaved for 20 min. The stock solution thus obtained was diluted with 4 parts of sterile distilled water before use for the preparation of antigen or as diluent in the CF test.

Anti Au serum. A serum from a patient with haemophilia A was used throughout. The CF titre of the serum was 1 : 32.

Serological methods. These and the materials used have been described in detail [11].

Preparation for electron microscopy. Tween 80-treated and control preparations were measured into tubes at 0.3 ml aliquots. To one of each duplicate, 0.1 ml of normal human serum, to the other the same volume of anti-Au serum, was added. The mixtures were kept at 4°C overnight. In the mixtures containing immune serum a precipitate appeared which was homogenized by intensive pipetting. A halving dilution series was prepared from each homogenate in isotonic ammonium acetate solution and an equal volume of 3% potassium tungstate (pH 7.6) was added to each dilution. One drop of each mixture was placed on a 200-mesh carbon-formvar-coated grid and the excess fluid was withdrawn by a filter paper strip. The preparations were examined in a Tesla BS 513A electron microscope.

Ultra-thin sections. To the two mixtures containing normal serum, buffered OsO_4 was added to reach a concentration of 1%. The mixtures were allowed to stand at +4°C for 1 hour. A well-visible precipitation occurred in the control tube, whereas in that containing Tween-80-treated material the precipitate was too little for further processing. The control precipitate was centrifuged at 2000 rpm for 10 min. The sediment was dehydrated without having been resuspended, and embedded in Durcupan ACM. Sectioning was carried out in a Reichert OM-U2 ultramicrotome and the sections were counterstained with lead citrate and uranyl acetate.

Results

Serological control of the preparative process. The Au antigen CF titre of the preparation in the different phases of processing is shown in Table I.

Table I

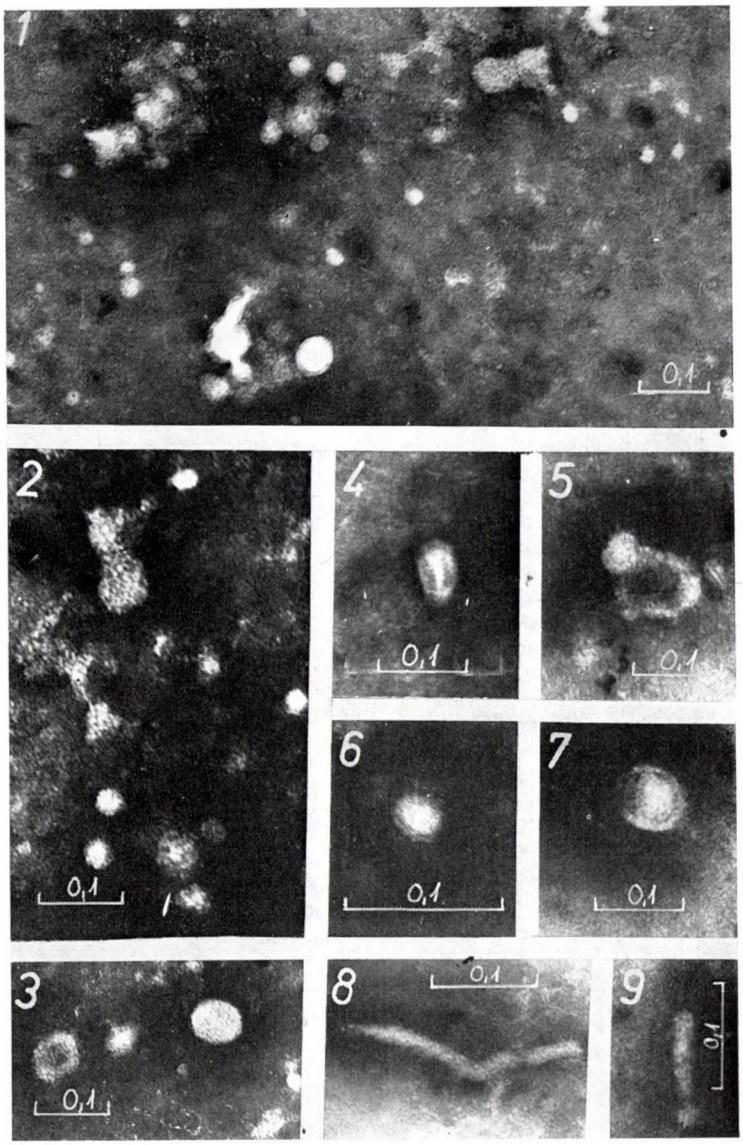
Au antigen CF titres of the different fractions

Fraction	CF titres in experiment	
	1	2
Clarifying centrifugation		
Supernatant	60	40
Centrifugation at 46 000 *g*		
Sediment	20	15
Supernatant	80	40
Centrifugation at 23 000 *g*		
Sediment	10	10
Supernatant	20	20
Centrifugation after Tween 80 treatment	8	4

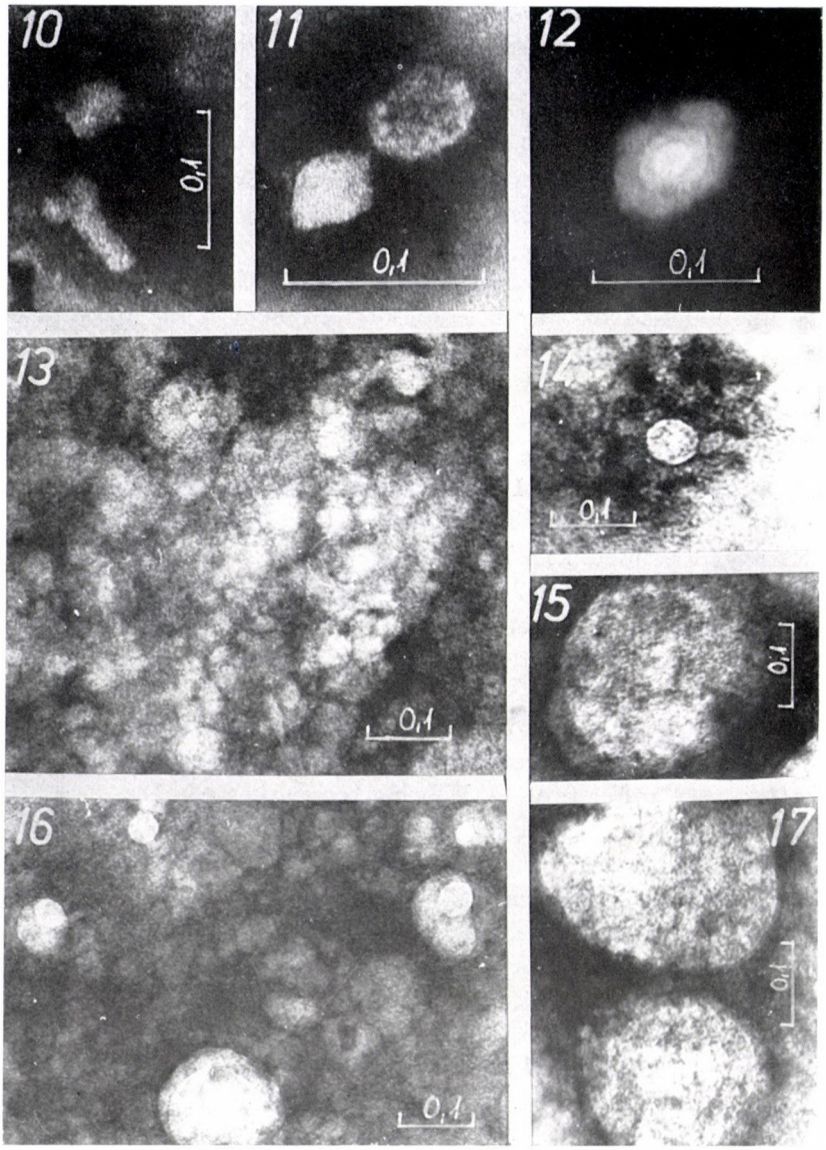
After centrifugation at 46 000 *g*, in the supernatant the Au antigen titre was practically equal to the initial titre; the titre of the sediment resuspended in 1/20th of the original volume was approximately one-third of that. Subsequent centrifugation at 23 000 *g* was of greater effect, probably due to the smaller volume and the earlier removal of lipids: two-thirds or a half of the antigenic activity was regained in this sediment. The titre of the supernatant showed no decrease as compared to that of the first sediment. Tween 80 treatment resulted in a further reduction in the Au antigen titre. It was reasonable to assume that the Au antigen recovered in the sediment did not represent the average Au antigen, but mainly its fraction(s) associated with particles of larger size and/or higher density.

Electron microscope studies. Since the two series of pictures were of approximately the same appearance no distinction between them will be made in the following.

Figs 1—12 show a negatively-stained Au antigen preparation not treated with Tween 80. The general appearance of the preparation is shown in Fig. 1. Most of the particles are 19 to 29 nm or 40 to 50 nm in diameter. Some of them show substructures, others appear empty. There are two larger particles near the bottom of the picture. Each has a well-defined limiting membrane and a compact electron-dense core. One of them is spherical in shape and 60 nm in diameter, the other is larger and irregular in shape. Part of the same photo



Figs 1 to 9. Purified Au antigen preparation. Negative staining (×120 000–270 000)



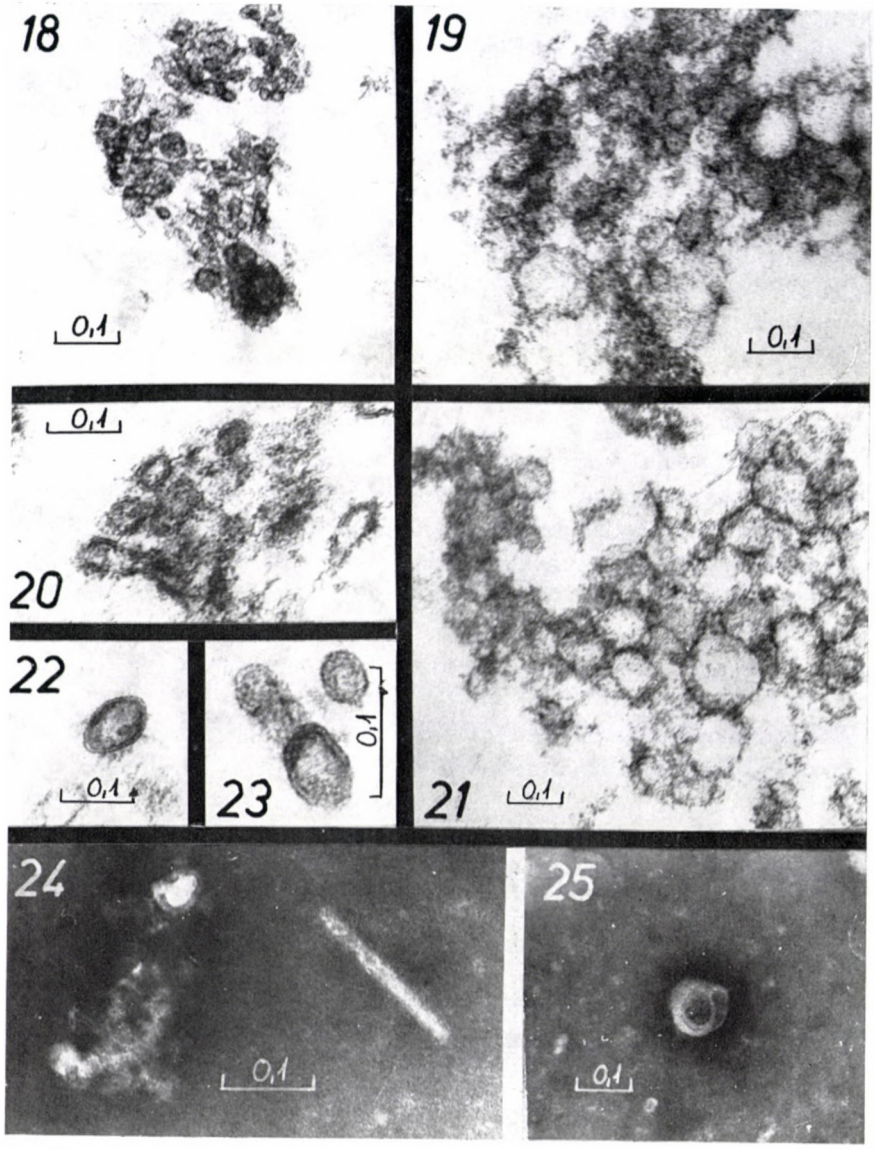
Figs 10 to 12. Purified Au antigen preparation. Negative staining (×270 000– 324 000)
Figs 13 to 17. Purified Au antigen mixed with haemophiliac A serum. Negative staining (×120 000–135 000)

is shown in Fig. 2. The somewhat larger magnification reveals the structure of the Dane particles. A Dane particle near the bottom appears to be disintegrating; its outer coat is injured and its core seems to be composed of globules of four different electron densities. A large irregular particle near the top, resembling a duck's head shows a filamentous structure on the "neck" and on the "beak" and a globular structure on the "head". Both particles in Fig. 3 are 75 to 80 nm in diameter. The left one reminds of a ring in shape. Both are filamentously substructured. The threads are about 5 nm in thickness. The particle in Fig. 4 is 45×90 nm in size. The vertical electron-dense stripe in its middle part shows a cross-striation of 8 nm periodicity. The particle has a distinct outer coat. The particle in Fig. 5 appears to have a tail, being partly cross-striated, partly globular in substructure. The one in the centre of Fig. 6, 25 nm in diameter, has an outer coat and an inner core, both substructured. Fig. 7 shows a particle with an inner core apparently helical in arrangement. In Figs 8 to 10 tubular particles very irregular in shape are seen. That in Fig. 10, resembling a budding mycelium, contains few electron-dense structured elements. In Fig. 11 a large oval particle 61×45 nm in diameter and a smaller angular one are seen. The former shows a globular structure, with globules 8 to 18 nm in diameter, filled up with an electron-dense substance, or empty in appearance. The angular particle shows a filamentous substructure.

Among the particles in Figs 1 to 12 many correspond to the 20 nm spherical particles, others seem to be identical with the Dane particles. Further particles, however, differ from both, being larger than the Dane particles and many (*e.g.* those shown in Figs 4, 6, 7 and 12) of them irregular in shape. It is questionable whether these and the particles with filamentous or globular substructure possess any Au antigen specificity.

With anti-Au serum the Au antigen preparation the morphology of which is shown in Figs 1 to 12 formed a precipitate, well visible to the naked eye. The negatively stained precipitate is shown in Figs 13 to 17. The particles in Fig. 13 are variable in size and shape. The diameter of the particles ranges from 16 to 65 nm. Each particle is surrounded by a mass of immunoglobulin molecules, even the background is covered by globulin threads. The spherical particle in the centre of Fig. 14, 52 nm in diameter, is also surrounded by filamentous structures. In the upper left corner aggregated smaller particles are seen. Fig. 15 shows a micro-drop of the precipitate containing mainly small particles. Those in Fig. 16 are more than 40 nm in diameter. The two micro-drops in Fig. 17 contain mainly 20 nm particles, in the upper one there are at least two particles of about 40 nm; all are embedded in immunoglobulin filaments.

It may be stated that antibodies in the haemophilia A serum bind to the surface of the particles under study, irrespective of their size.



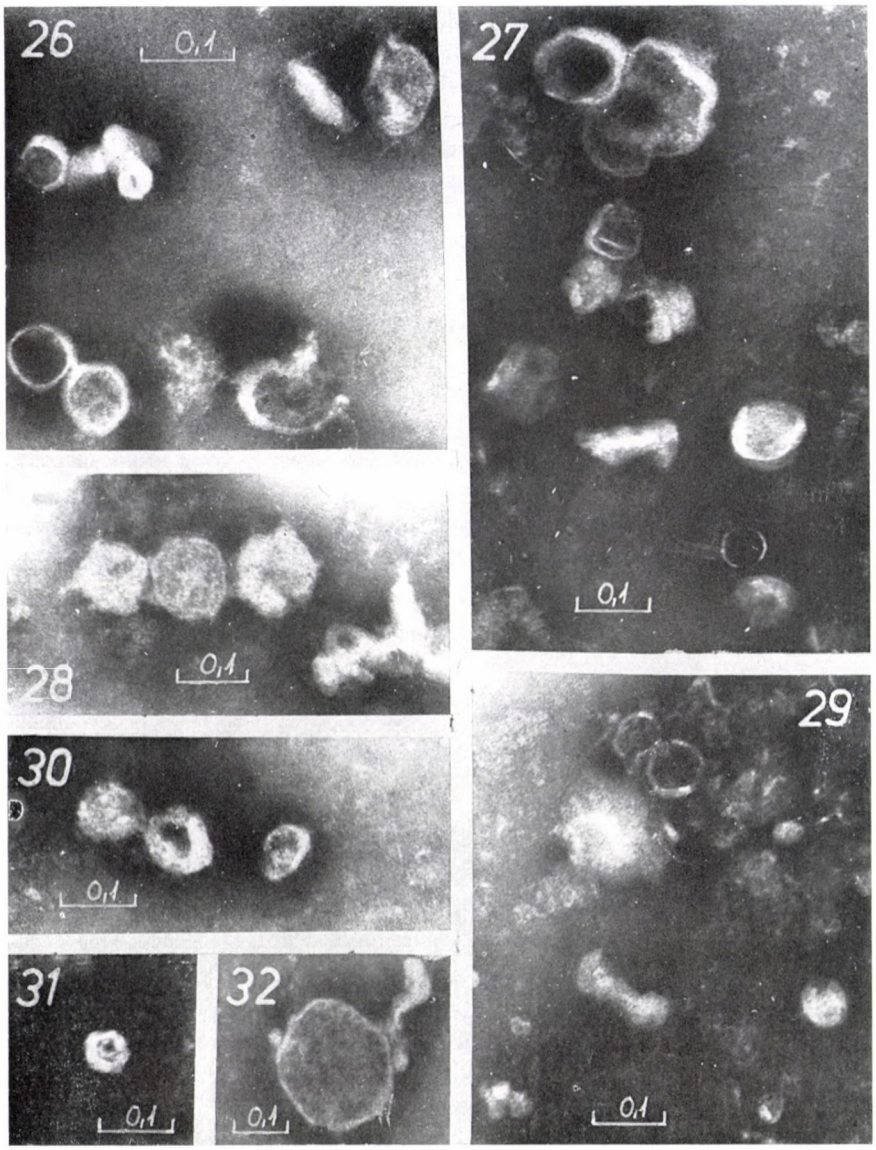
Figs 18 to 23. Purified Au antigen. Ultra-thin sections (×90 000–216 000)
Figs 24 and 25. Purified Au antigen treated with Tween 80. Negative staining (×120 000–150 000)

Figs 18 to 23 show ultra-thin sections of the preparation shown in Figs 1—12. Most of the particles in Fig. 18 are 16 to 24 nm in diameter, having no electron-dense core. Some 24 nm or larger particles appear to be filled with an electron-dense substance. The particles in Fig. 19 are mainly of the same range of magnitude except some smaller ones near the upper right corner; at least three particles approximate 90 nm in diameter. These apparently empty particles are covered by a structured membrane. Typical Dane particles are shown in Fig. 20. Five of them are limited by unit membrane and contain different amounts of a substance of variable electron density and granular or filamentous in structure. The particles in Fig. 21 are in general larger but variable in size, most of them are limited by a simple membrane and poor in inner structure. The particles in Figs 22 and 23 are 25 to 80 nm in diameter, have unit membranes and a globular or filamentous inner structure.

In the ultra-thin sections, the particles are polymorphous both in size and structure. Only few of them are provided with a unit membrane, and a structured electron-dense inner core, characteristic of virions. These particlesrange from 25 to 60 nm in diameter.

Preparations shown in Figs 24 to 32 were subjected to Tween 80 treatment for 30 min. Efforts were made to find particles resembling those shown in Figs 1 to 23 and those considered typical by ALMEIDA *et al.* [9]. The most characteristic findings are shown in the figures.

The rod-shaped particle in Fig. 24 is less substructured and slimmer than are the corresponding untreated particles in Figs 8 to 10. There is a Dane particle in the upper part of the same picture. Its core appears to be more compact and its outer coat is unimpaired (for comparison, see Fig. 6). The amorphous substance below the Dane particle may consist of a disintegration product like that shown in Figs 2, 3 and 11. The particle in Fig. 25 is the most similar to the Tween-80-treated particles published by ALMEIDA *et al.* [9]. (For comparison, see Figs 1 and 7.) Supposedly, only particles provided with a unit membrane show this type of impairment resulting in revelation of the inner surface. Part of the core material had probably been released. In Fig. 26, the membrane of the particle near the lower right corner seems to be thickened; it had probably shortened because of the shrinkage or outflow of its content. The outer coat is thickened in the other particles as well. These particles seem to be partly or completely empty. There is some globular inner structure in the particle in the upper right corner. Due to the impairment of the particles, their diameter had changed. In Fig. 27 there are some new elements: from the bottom upwards, the second particle is empty and has no coat structure. A little below the centre of the picture, the outer coat of a filamentous particle has rarified; its core appears to have condensed. Two very large particles near the top of the picture have no inner core. The particle near the centre of Fig. 28 shows an outer coat which is more pronounced than that of the un-



Figs 26 to 32. Purified Au antigen treated with Tween 80. Negative staining (×120 000–150 000)

treated particles; its core, on the other hand, has rarified. In contrast, the two other particles show a more pronounced filamentous structure and no sign of emptying. In Fig. 29, there are several apparently intact particles 20 to 25 nm in diameter and a disintegrated inner core. A large particle and a filamentous one below it show a similarly altered structure. The ring-shaped form in Fig. 30 reminds of a structure in Fig. 3. Here the Tween 80 treatment caused no characteristic changes. In the two other particles both globular and filamentous structures occur. The Dane particle in Fig. 31 shows a well-defined impairment. The membrane of the large form in Fig. 32 is injured at some places, elsewhere it is of increased intensity.

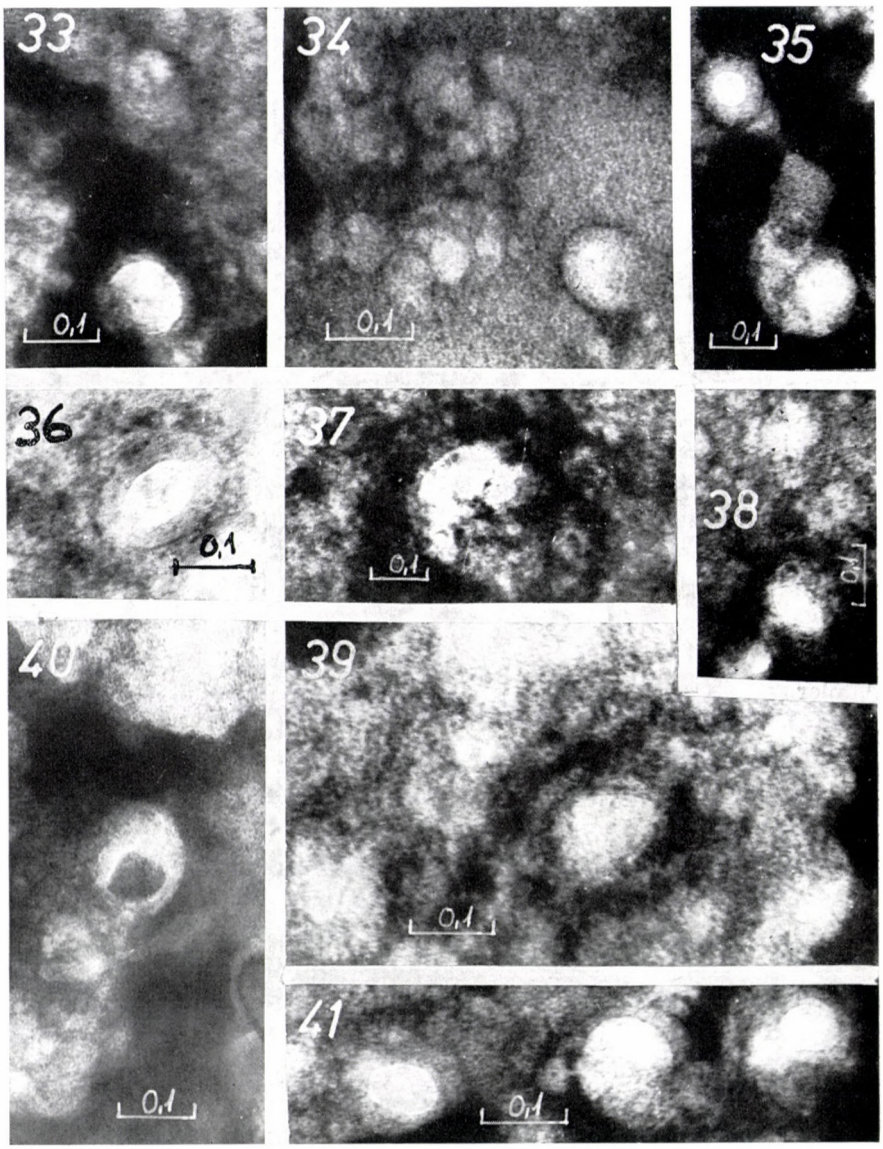
Figs 24 to 32 show that the Tween 80 treatment caused variable changes in the structure of the different particles. Those provided with unit membrane were impaired and, consequently, a new surface became free in them. In other particles, whether spherical or filamentous, the core became more pronounced.

The exposure of new surfaces has prompted us to examine the antigenic specificity of the modified particles. For this purpose the Tween-80-treated preparation was mixed with our anti-Au serum. The negatively stained preparations are shown in Figs 33 to 41.

According to ALMEIDA *et al.* [9] the anti-Au sera of patients with haemophilia A contain antibodies to the inner-core antigen as well.

The outer coat as well as the denuded left side of the large particle at the bottom of Fig. 33 is covered by antibody molecules. A similar phenomenon is seen on the large particles in Figs 37 and 39. All the other corpuscles rather variable in size, shape and structure (including two ring-shaped forms similar to that seen in Fig. 27) are surrounded by antibody masses. The particles in Fig. 34 have no outer coat; they may represent small particles, released inner cores or precipitates of the latter. In Fig. 35 there are two particles of about 40 nm, possessing no distinct outer coat. The substance between them appears to be a precipitate consisting of disintegrated antigenic substance and immunoglobulins. Fig. 36 shows an apparently intact particle embedded in precipitate. Although the orientation in Fig. 38 is difficult, there is no doubt that an immunoprecipitate of particles extremely different in size is seen. Fig. 40 shows a partially emptied virus membrane. Both the membrane and the discharged substance are surrounded by an antibody mass. Each of the three particles in Fig. 41, highly different in diameter, has membrane-covered and membrane-free surfaces all covered by antibody molecules.

This last group of electron micrographs indicates that our anti-Au serum of human origin contained antibodies to different components of Au antigen particles. The intensive immunoprecipitation in preparations of low Au antigenicity should be noted.



Figs 33 to 41. Purified Au antigen treated with Tween 80 + haemophiliac A serum. Negative staining (×120 000–160 000)

Discussion

In sediments obtained by relatively low-speed centrifugation, the proportion of the larger particles and of those of higher specific gravity is increased. For this reason our preparations were rich not only in Dane particles but also in still larger corpuscles, 60 nm or more in diameter, at the expense of the 20 nm Au antigen particles. It has been shown that the selected preparation was poor in CF antigenic activity. The bulk of this activity remained in the supernatant.

The question arises whether a pure suspension of large particles would be devoid of CF antigenic activity. The data in Table I appear to support this assumption. However, the electron micrographs have convinced us that particles variable in size and inner structure bind antibodies from the anti-Au serum. Accordingly, these sera contain antibodies which bind to particles of variable morphology, very poor in Au-specific CF antigenic activity. It might be supposed that the morphologically different particles are of the same serological specificity, the only difference being that the reaction with the large particles binds less complement. As another possible explanation, the particles of different size may have different antigenic component(s) in addition to common ones. Of course, removal of the bulk of the small-sized particles with the supernatants may be responsible for a considerable part of the lost CF antigen.

We suppose that the morphologically more complex large particles bear more different antigenic determinants than the small particles. This might be in agreement with the finding of ALMEIDA *et al.* [9] that hepatitis convalescent sera, even those containing no demonstrable anti-Au antibodies, may react with some inner antigenic factor of the Dane particles. If so, these antibodies may play a considerable role in the course of hepatitis.

Among the particles encountered in the present study, Dane particles, which represent a very small proportion of particles in the Au-antigen-positive fractions, are the most similar to infectious virions. According to MELNICK's estimation [cit. 12], 1 ml Au-antigen-positive serum contains 10^{10} to 10^{11} small particles. In spite of this, the infectivity of such sera proved to be of very low titre in the experiments of KRUGMAN *et al.* [13, 14]. There is little reason to assume that the sometimes very large particles limited by a simple membrane might be infectious. The most probable is that among the particles of different morphology occurring in the Au-positive sera the typical Dane particles represent the infectious virus.

Tween 80 treatment of the preparations resulted in an impairment of most of the typical Dane particles; consequently their inner cores were partly released. The same occurred to some of the still larger particles: the limiting membrane of these particles increased in density. In our material the ratio

of 20 nm particles to larger particles appeared to decrease on Tween 80 treatment, perhaps for technical reasons.

It was interesting that the rarified inner structure of some particles became more distinct under the effect of the reaction with anti-Au serum. The fact that these particles retained their serological activity, together with the low CF antigen activity of these sera, allows to assume that the activity of the Tween-80-treated particles in immuno-electron microscopy may partly be due to antibodies different from anti-Au antibodies directed against the outer part of the particles. Sera from haemophiliacs, like the specimen used by us, may contain antibodies to different specificities of the hepatitis B virus.

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ISOLATION OF MYCOPLASMA PNEUMONIAE FROM HUMAN PRIMARY ATYPICAL PNEUMONIA

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Summary. *Mycoplasma pneumoniae* showing characteristic biochemical and serological properties was isolated from the sputum of 3 patients with primary atypical pneumonia. The strains were sensitive to erythromycin, tetracycline and oleandomycin.

Isolation of *Mycoplasma* from human materials has been reported by a number of workers [1—3]. On the basis of serological examinations [4] 3 different species were established: *M. hominis*, *M. salivarum* and *M. fermentans*. Later, strains of various origin have been isolated which differed from the above organisms [5—23]. Of these, *M. pneumoniae* is of special importance, being undoubtedly a pathogenic species. In this paper, studies on 3 strains are described.

Materials and methods

Cultivation. Sputum of hospitalized patients with pneumonia reacting unsatisfactorily to treatment was inoculated into liquid mycoplasma medium; heart infusion broth (Difco) prepared as specified, 90 ml; inactivated horse serum, 20 ml; fresh Hayflick yeast extract, 10 ml; thallium acetate (BDH Chemicals, England) 10% aqueous solution, 1 ml; desoxyribonucleic acid (Sigma) 0.2% aqueous solution, 1.2 ml; glucose 50% aqueous solution, 1 ml; phenol red 0.006% aqueous solution, 5 ml; vancomycin, 0.25 mg per ml medium; penicillin, 200 000 IU per ml solution, 0.25 ml. A serial tenfold dilution of the sputum was prepared in the above medium in the range of 10^{-1}—10^{-5}. Incubation lasted for 45 days at 37°C. At 2–3 day intervals, subcultures were made on a solid medium containing the same ingredients. The plates were incubated in wet chamber at 37°C for 45 days and examined weekly under a stereomicroscope. Mycoplasma colonies were transferred into broth, then the culture was filtered through Millipore membrane (0.22 μm pore size). The filtrate was spread over solid plates. From all isolates two colonies were subjected to three consecutive subcultures by picking up isolated colonies. Cultures obtained in this manner were freeze-dried and tested for biochemical and serological reactions.

If the primary liquid and solid medium cultures showed no evidence of growth, blind passage from broth to broth was performed at weekly intervals. After three unsuccessful passages the material was regarded as negative.

For biochemical reactions shown in Table I see the following references; glucose [24], sodium taurocholate and sodium polyanethol sulphonate sensitivity [25], phosphatase activity [26], aesculin hydrolysis [27], 2,3,5-triphenyltetrazolium chloride and methylene blue reduction [28], cholesterol requirement [29].

Serological examination. Growth inhibition [14] and fermentation inhibition [13] tests were carried out with immune sera prepared against the following strains: *M. pneumoniae* (Mac), *M. salivarum* (PG20), *M. fermentans* (PG18), *M. arthritidis* (PG6), *M. orale* I (CH 19 299), *M. orale* II (CH 20 247), glucose splitting animal strains *M. bovirhinis* (NCTC 10 118), *Mycoplasma* sp. (PG49, Squire), *A. laidlawii* (NCTC 10 116), *M. granularum* (NCTC 10 128), *M. neurolyticum* (PG28), *M. gallisepticum* (S_6). Patient sera were examined also with the growth and fermentation inhibition tests.

Antibiotic sensitivity. Antibiotic discs prepared by the Institute for Serobacteriological Production and Research Human, Budapest, were used. Actively growing cultures were diluted 10^{-1}—10^{-5} and stored at —20°C. The number of c.f.u. was determined after thawing the suspension. For antibiotic sensitivity testing the frozen stock dilution showing 10^5 c.f.u. was melted and plated.

Results

Three out of 4 patients with primary atypical pneumonia were shown to excrete mycoplasma in their sputum. None of the patients had had any connection with one another. In two cases mycoplasma appeared on the 15th day on plates inoculated from 6—7-day broth cultures as a few tiny colonies.

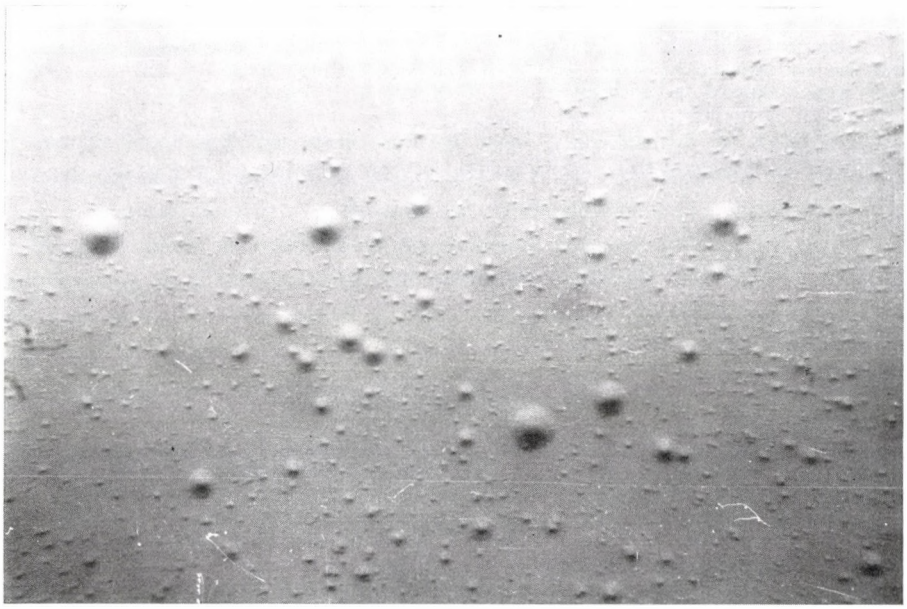

Fig. 1. M. pneumoniae strain 565, 5 days (×25)

In the third case tiny colonies without peripheral zone were observed on plates seeded on the 10th day from the second broth passage. Broth cultures shown to contain mycoplasma became yellow in colour in 6—7 days after plating on solid medium. In further passages the broth cultures showed a yellow colour in 3 days and plate cultures derived from these tubes were characterized by colonies with peripheral zone (Fig. 1). The organism was recovered from primary tubes containing the sputum in 10^{-1} dilution in two cases and in 10^{-2} dilution in one case.

Biochemical tests are shown in Table I. The 3 strains behaved uniformly, while there was some difference in the intensity of certain reactions. For the aesculin test, 1% PPLO serum fraction was unsuitable as a substitute for horse serum.

Table I

Properties of mycoplasma isolates

	565	566	568
Glucose, aerobic	+	+	+
Glucose, anaerobic	—	—	—
Mannose	+	+	+
Mannitol	—	—	—
Sucrose	—	—	—
Galactose	—	—	—
Xylose	—	—	—
Aesculin	—	—	—
Arbutin	—	—	—
Arginine	—	—	—
Urea	—	—	—
Cholesterol requirement	+	+	+
Growth without serum	—	—	—
Liquoid sensitivity	6.0	6.0	6.0
Digitonin	4.0	4.0	3.0
Tetrazolium reduction, aerobic	+	+	+
Tetrazolium reduction, anaerobic	+	+	+
Methylene blue reduction, aerobic	—	—	—
Methylene blue reduction, anaerobic	+	+	+
Film and spot	—	—	—
Phosphatase	—	—	—

Growth inhibition test was positive only with *M. pneumoniae* antiserum (Table II). In the fermentation inhibition test *M. pneumoniae* (Mac) antiserum inhibited the isolates at 1 : 80—1 : 160 dilution.

Table II

Growth inhibition test for mycoplasma isolates with reference sera prepared against glucose positive human mycoplasma species

Reference immune sera	Strains isolated from patients			Reference strains	
	565	566	568	*M. pneumoniae* (Mac)	*M. fermentans* PG 18
M. pneumoniae (Mac)	6.5	3.5	4.0	7.5	—
M. fermentans PG 18	—	—	—	—	4.0

Growth inhibition test with the patient sera is presented in Table III. The same sera in the fermentation inhibition test showed titres against reference strain *M. pneumoniae* (Mac) as follows: 1 : 10 for patient harbouring strain 565, 1 : 80 for 568 and 1 : 20 for 566.

Table III

Growth inhibition test for mycoplasma isolates with patient sera

Organism	Serum of patient		
	565	566	568
Strain 565	1.0	2.5	1.5
Strain 566	0.5	2.5	3.0
Strain 568	1.0	3.0	1.5
M. pneumoniae (Mac)	1.0	3.0	1.5

Table IV

Antibiotic sensitivity of mycoplasma isolates

Antibiotic disc	Zone of inhibition, mm		
	strain 565	strain 566	strain 568
Chloramphenicol	25	14	10
Streptomycin	14	—	—
Oleandomycin	33	30	30
Erythromycin	31	31	30
Tetracycline	30	23	21
Oxytetracycline	26	26	22
Neomycin	21	29	16
Kanamycin	20	17	8*
Spiramycin	24	24	10*
Novobiocin	20	12	11

* Slight growth within the inhibition zone.

Antibiotic sensitivity of the strains is shown in Table IV. It is seen that the zones of inhibition were fairly uniform for the 3 strains, differences appeared only in streptomycin, kanamycin and spiramycin sensitivity. All isolates were sensitive to erythromycin and oleandomycin.

Discussion

The mycoplasma strains isolated in the present study corresponded in biochemical characteristics to the properties of *M. pneumoniae* as described by ALUOTTO *et al.* [24] and in serological behaviour to *M. pneumoniae* type

strain Mac. Their aetiological role in primary atypical pneumonia seems to be confirmed by the results of serological tests performed with the patient sera. The results indicate that in Hungary mycoplasma infections should be taken into consideration and laboratory examinations for detecting the causative agent should be extended. According to the present observations, erythromycin seems to be the antibiotic of choice for the treatment of primary atypical pneumonia.

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A BACTERIAL SYSTEM USEFUL IN THE ISOLATION OF RNA SYNTHESIS STIMULATING SUBSTANCES FROM PHYTOHAEMAGGLUTININ OR FROM SERA

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Summary. *Escherichia coli* Q13 cells plasmolyzed in 0.3 M sucrose have a low basic metabolism, an increased permeability, and are characteristically stimulated to increase RNA synthesis by a factor (F V) isolated from red kidney beans and by sera or serum protein fractions. The RNA synthesis in these cells is sensitive to rifampicin and to actinomycin D. The optimum concentration of K^+ for RNA synthesis in plasmolyzed *E. coli* cells is 2 mM at 0–50 mM NH_4^+ concentrations. Thiols suppress the RNA synthesis of unstimulated cells. In the presence of mercaptoethanol this depression is counteracted by F V, giving a high stimulated/background ratio of RNA synthesis. Plasmolyzed *E. coli* cells, incubated in the presence of 0.3 M sucrose, 2 mM K^+, 50 mM NH_4^+, and 5 mM mercaptoethanol (and some additional components) present a simple, rapid system for studies of stimulators and inhibitors, of RNA, DNA, and protein synthesis. The system described seems to be a useful and simple biological guide for the extraction and separation from biological material of different activators or inhibitors of nucleic acid and protein synthesis.

Recently we have described the use of a bacterial system as a biological guide in work aiming at the separation of RNA synthesis stimulating substances in phytohaemagglutinin (PHA) from red kidney beans [1].

The system consisted of *Escherichia coli* cells plasmolyzed in the middle log phase in hypertonic sucrose solution. The plasmolyzed *E. coli* cells have an increased permeability and a low metabolic activity. They may be considered resting cells which respond promptly with an increased RNA synthesis to the addition of certain stimulators isolated from PHA.

In the present communication, some characteristics of this bacterial system are described, especially with regard to the components of the incubation medium affecting the rates of RNA and protein synthesis in the resting and in the stimulated plasmolyzed cells.

Materials and methods

Chemicals. [5-^{3}H]Uridine, [methyl-^{3}H]thymidine, and [ring-4-^{3}H]L-phenylalanine from Radiochemical Centre (Amersham); casamino acids from Difco; rifampicin from CIBA; actinomycin D from Nutritional Biochemicals Co.; inorganic chemicals and chemicals of analytical grade from Merck (Darmstadt); all other chemicals and biochemicals were purchased from Sigma Chemical Co. Factor F V from *Phaseolus vulgaris* seeds was prepared as described by HARMS-RINGDAHL *et al.* [1].

Assay for stimulation of RNA, protein, or DNA synthesis. The system consists of *E. coli* cells subjected to plasmolysis. Treatment of the cells is related to the procedure applied by

HEPPEL [2, 3] in order to increase permeability of the bacterial cell wall. *E. coli* Q13 (kindly provided by Dr. J. T. AUGUST, Albert Einstein College, Bronx, N.Y.) was grown in a medium containing $Na_2HPO_4 \cdot 2H_2O$, 8.8. g; KH_2PO_4, 3.0 g; NaCl, 0 5 g; NH_4Cl, 1.0 g; glycerol, 3.0 g; casamino acids (technical), 6.0 g; $MgSO_4 \cdot 7H_2O$, 1.25 g per litre solution. The medium was sterilized by filtration through a 0.22 μm Sartorius filter. The culture was aerated by shaking at 37°C until bacterial growth reached the middle log phase, *i.e.*, a density of 10^8 cells per ml. The suspension was then cooled rapidly to 0°C and the bacteria collected by centrifugation at +2°C for 15 min at 12 000 *g*. The cells were suspended in the original volume of 0.03 M Tris buffer pH 8.1 at 0°C, and after 5 min slow shaking at 0°C the centrifugation step was repeated. The collected cells were then resuspended at 0°C in a SMG solution containing 10% (w/v) sucrose, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% glucose, to yield a cell suspension of 6.0×10^8 cells per ml. The cell suspension was stored at +2°C and used for experiments after 12 hr and up to an age of one week. During storage for one week, the rate of incorporation of uridine usually dropped by a factor of 2, although stimulability remained practically unchanged.

Before use an aliquot of the suspension was centrifuged as above, the supernatant discarded, and the cell button resuspended at 0°C in fresh SMG solution to yield a standard bacterial suspension of 3.0×10^7 cells per ml.

In order to measure the stimulation or inhibition of RNA, protein or DNA synthesis, the cells were incubated together with the factor to be assayed at 35°C in the presence of a suitable reaction mixture containing either [^{3}H]uridine or [^{3}H]phenylalanine or [^{3}H]thymidine, and the incorporation of these precursors was measured. The reaction mixture (RM), derived from the one generally used in cell free protein synthetizing systems, was freshly prepared daily.

The composition of RM was Tris-Cl buffer, 0.112 M pH 7.8; NH_4Cl, 0.1 M; KCl, 3.7 mM; Mg acetate, 2.6 mM; ATP (Na salt), 2.5 mM; GTP (Na salt), 0.6 mM; creatine phosphate (Na salt), 26 mM; creatine phosphokinase, 0.13 mg/ml; sucrose, 0.34 M; uridine, 0.015 mM; each of nineteen amino acids (except phenylalanine), 0.07 mM; phenylalanine, 0.015 mM; thymidine, 0.015 mM; 2-mercaptoethanol, 10 mM; and either [^{3}H]uridine 7.5 μCi/ml (24 Ci per mmole) or [^{3}H]phenylalanine 7.5 μCi/ml (18 Ci per mmole) or [^{3}H]thymidine 7.5 μCi/ml (14.1 Ci per mmole). The incubation mixture for one experiment consisted of 134 μl RM to which was added the sample to be tested dissolved in 66 μl distilled water. Incubation was started by adding 50 μl of the standard bacterial cell suspension in SMG solution and by placing the tube in a 35°C water bath. At different times an aliquot of the incubated mixture was mixed with an equal volume of 10% (w/v) sodium dodecylsulphate (SDS) and incubated at 35°C for 15 min. When the cells were lyzed, 50 μl was transferred to the middle of a glass-filter (Whatman GF/C, 2.5 cm). The filter was dried for 1 min at room temperature and immersed in ice-cold 10% (w/v) trichloroacetic acid (TCA). When investigating amino acid incorporation, the filters were further processed in order to hydrolyze aminoacyl-tRNA and to eliminate non-incorporated radioactivity according to the procedure of MANS and NOVELLI [4]. When the incorporation of uridine or thymidine was measured, the hydrolysis with hot TCA was omitted and the filters were washed several times with 5% ice-cold TCA, then with alcohol and ether. Finally, the filters were dried, placed in scintillation vials, and incubated at 55°C for 30 min with 0.5 ml toluene (TM 100, Packard). Then 10 ml of scintillation fluid (0.5% PPO and 0.005% POPOP in toluene) was added and radioactivity was measured in a nuclear Chicago Liquid Scintillation Counter Model 6850 with an efficiency of 19%. The data obtained were expressed as pmole uridine (or thymidine or amino acid), incorporated in 10^6 cells.

Since the rate of nucleic acid and protein synthesis decreases in the plasmolyzed cells due to ageing, furthermore the successive cell preparations also differ somewhat in synthetic activity, the data collected in a Figure or Table derive from one and the same experiment. Within one and the same experiment the counting error and the experimental error give a 95% confidence interval amounting to 10% of the determined incorporation.

Results

1. *Time course of RNA, DNA, and protein synthesis.* The stimulability of the plasmolyzed bacterial cells is demonstrated in Fig. 1 which shows the time course of RNA, DNA and protein synthesis measured by the incorporation, after different times, of labelled uridine, thymidine and phenylalanine added at the start of the experiment.

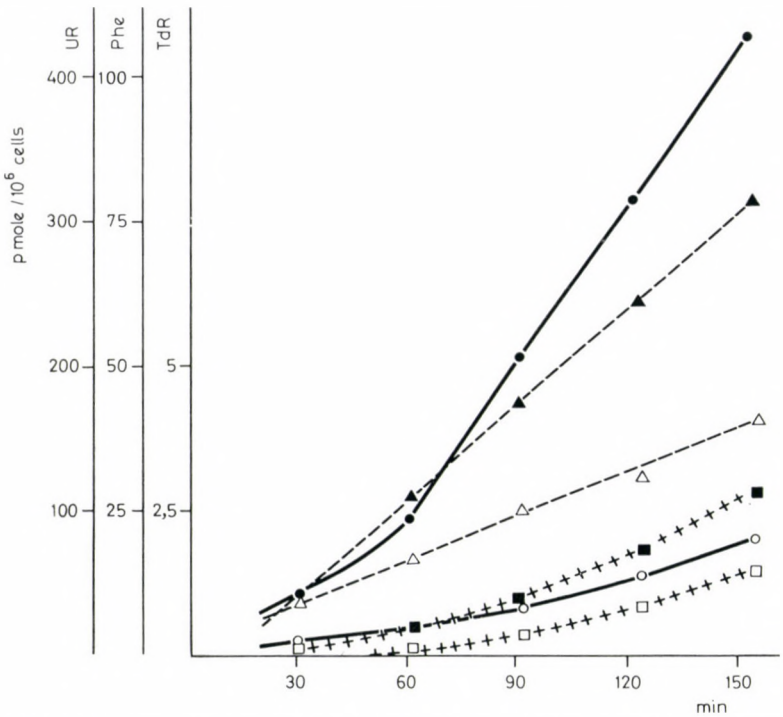

Fig. 1. RNA, DNA and protein synthesis in *E. coli* cells as measured by the incorporation of uridine (UR), thymidine (TdR) and phenylalanine (Phe) during different periods of incubation from the start of the experiment. The synthetic activities in the absence ("background level") and presence of the activator F V (6 μg N/ml test) *Phaseolus vulgaris* seeds are given.
UR: —F V ○———○; Phe: —F V △— — —△; TdR: —F V □+++□,
+F V ●———●, +F V ▲— — —▲, +F V ■+++■

Figure 1 shows the incorporation of precursors in unstimulated cells and the incorporation after addition at time 0 of the stimulating factor, F V, from *Phaseolus vulgaris* seeds [1]. In this experiment, due to the stimulator, RNA synthesis rate increased by a factor of 5. The rates of protein synthesis and DNA synthesis were practically doubled.

2. *Influence of cations K^+ and NH_4^+.* The effect on RNA synthesis of K^+ and its interaction with NH_4^+, added as NH_4Cl, is shown in Fig. 2. In the absence of NH_4^+, addition of K^+ elicited an about twofold increase of RNA synthesis at the optimum (10 mM) K^+ concentration. NH_4^+ at 50 mM concentration strongly suppressed RNA synthesis at low K^+ concentrations and induced a shift of the optimum K^+ concentration from 10 mM to about 2 mM. At 100 mM NH_4^+, RNA synthesis was suppressed at all K^+ concentrations.

The interaction of K^+, NH_4^+ and added stimulator (F V) was investigated in a study of the stimulability of the system by F V in the presence (2 mM) and absence of K^+ at 0, 50, and 100 mM NH_4^+ (Table I). The greatest response

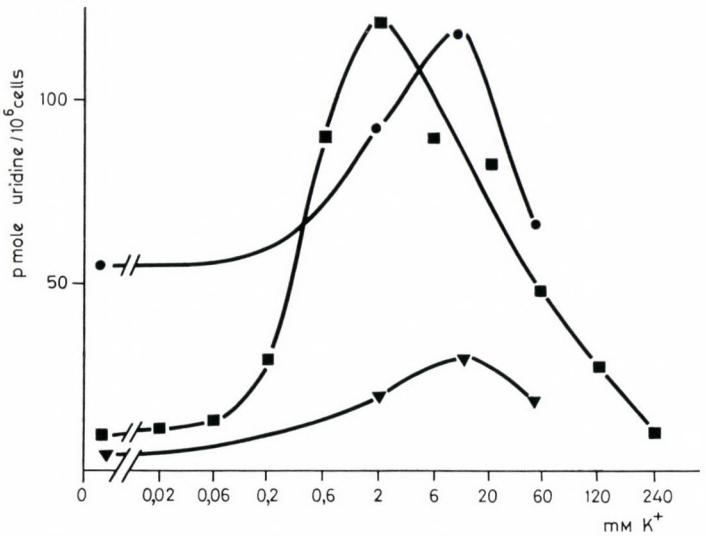

Fig. 2. Influence of K^+ and NH_4^+ on RNA synthesis in the absence of F V. Incorporation of uridine was measured after 150 min incubation. KCl and NH_4Cl were either omitted from, or added at different concentrations to, the standard reaction mixture (RM) described in Materials and methods. No NH_4^+ ● , 50 mM NH_4^+ ■ , 100 mM NH_4^+ ▲

Table I

Influence of K^+ and NH_4^+ on the stimulability of the E. coli system by F V

Five μg N of F V/ml test suspension. Incorporation of uridine was measured after 150 min incubation. KCl and NH_4Cl were either omitted from or added at different concentrations to the standard reaction mixture

NH_4^+ mM	pMole incorporated uridine/10^6 cells (in parentheses, per cents of the respective control)			
	K^+ absent		K^+ present (2 mM)	
	No stimulant	+F V	No stimulant	+F V
0	55	436	92	535
	(100)	(800)	(100)	(580)
50	2.7	141	117	387
	(100)	(5200)	(100)	(330)
100	1.9	19	20	202
	(100)	(1000)	(100)	(1000)

to F V was obtained in the absence of K^+, especially in the presence of 50 mM NH_4^+. F V produced a conspicuous partial reversion of the system's suppression by 100 mM NH_4^+ in the presence of K^+.

3. *Influence of thiols.* The incubation medium for the cells contains 5 mM mercaptoethanol (see Materials and methods), which induces a suppression of the system overcome by stimulators, at least of the type represented by F V. Mercaptoethanol was compared with a few thiols and cysteamine with respect to the influence on the bacterium system and on the stimulating factor F V. Fig. 3 summarizes three experiments (1, 2, 3) on the action of these compounds on the cell's RNA synthesis.

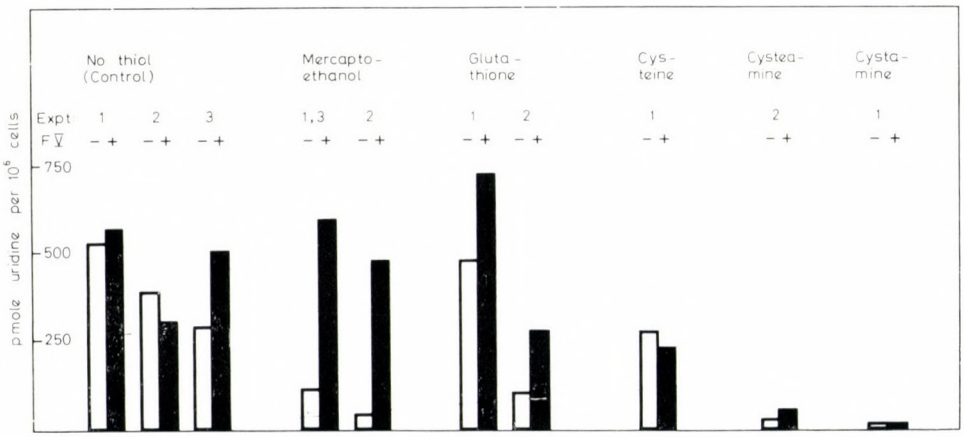

Fig. 3. Influence of thiols on RNA synthesis during 150 min in non-stimulated cells and in cells stimulated by F V (21.5 μg N/ml test). Mercaptoethanol was excluded from the RM and replaced by the respective thiols indicated in the diagram; 1, 2, 3 refer to three independent experiments. In each pair of columns the right one represents a test with F V, the left one a test with the same amount of distilled water. In experiment 1, F V and the thiol were preincubated at 20°C for 1 hr at 10 times higher concentrations than those of the test and at the onset of the test an aliquot of the pretreated F V was transferred to the mercaptoethanol free test system to a final thiol concentration of 5 mM. In experiment 2, unreduced F V (right columns) or the same volume of water (left columns) were added to test systems containing no thiol (control), mercaptoethanol (5 mM), reduced glutathione (2.4–20 mM, giving the same result), or cysteamine (2.4 mM; 20 mM being toxic). In experiment 3, F V was pretreated with 50 mM mercaptoethanol which was subsequently removed. The thiol-free controls were thus tested with unreduced F V in experiments 1 and 2, and with pretreated F V in experiment 3. As expected, experiments 1 and 3 with 5 mM mercaptoethanol in the test medium gave the same result. The controls (no thiol and without F V) of experiments 1, 2 and 3 show the variation in uridine incorporation with increasing age of the bacteria (cf. Materials and methods). Due to this variation quantitative comparisons should be done with the control of the same experiment

F V has to be in a reduced state (see Experiment No. 3, "no thiol", in contrast to No. 1, 2) in order to be active without reductant added to the system. Mercaptoethanol depressed RNA synthesis in the system, but a comparison with the thiol-free controls showed that F V was able not merely to counteract this inhibition, but even in the presence of mercaptoethanol it stimulated RNA synthesis of the system to the saturated level. F V pretreated with glutathione allowed an appreciable stimulation. In the presence of cysteine,

at about 50% reduction of the control RNA synthesis, no stimulation was obtained. Cysteamine which strongly inhibits RNA synthesis, allowed a certain stimulation, and cysteamine inhibited the system completely.

4. *Influence of proteins.* A few proteins were tested for their ability to stimulate plasmolyzed *E. coli* (Table II). At comparable concentrations,

Table II

Effects of proteins and a tissue culture medium on uridine incorporation in E. coli Q13 cells

Duration of the test, 150 min

Factor added	Final concentration (per ml)	Effect on RNA synthesis (pmole incorporated uridine per 10^6 cells)	
		Without F V	With F V (6 μg N/ml)
None	—	43	350
Dialyzed calf serum	60 μl	378	348
(Microbiological Associates, Bethesda, Md.)	20 μl	182	236
Bovine serum albumin (Sigma)	1.6 mg	269	384
Bovine serum albumin (Sigma) heated to 60°C for 30 min	1.6 mg	285	381
Blood albumin (British Drug Houses)	1.6 mg	420	447
Human Serum Albumin (Kabi, Stockholm)	1.6 mg	81	307
Casein (Hammarsten, Merck)	1 mg	42	276
MU medium*	0.1 ml	71	306

* A mixture of Medium 199 and Waymouth Medium, as described by HARMS-RINGDAHL *et al.* [1], without penicillin, streptomycin and [^{3}H]uridine.

dialyzed calf serum, bovine serum albumin (BSA) and blood albumin had a significant stimulating effect on RNA synthesis. In the case of BSA, this activity was unchanged after heating to 60°C for 30 min. A slight inhibitory effect on the action of F V was found for calf serum. A cell culture medium (MU), consisting of Parker 199 and Waymouth MB 752/1 (Flow), used in our studies on the stimulation of chicken spleen lymphocytes [1] and human serum albumin had a slight effect on plasmolyzed *E. coli* cells and permitted a good stimulation by F V. Caseine had no stimulating activity.

5. *Effect of RNA synthesis inhibitors.* The action on RNA synthesis of the stimulator F V from *Phaseolus vulgaris*, and of the inhibitors rifampicin and actinomycin D added at different concentrations to the incubation medium is shown in Table III. The experiment demonstrates a strong response to F V, a high sensitivity to rifampicin and a remarkable inhibition by actinomycin D.

Table III

Effects of the stimulator, F V, and of inhibitors on RNA synthesis

The incorporation of uridine during 150 min is given in per cents of the control value (45 pmole/10^6 cells = 100%)

F V		Rifampicin		Actinomycin D	
concentration	effect	concentration	effect	concentration	effect
(μg N/ml)	(%)	(μg/ml)	(%)	(μg/ml)	(%)
0.64	180	2.3	26	3	100
3.2	590	4.5	17	12	75
16	870	9	4	24	52

Discussion

1. *Some characteristic properties of plasmolyzed E. coli cells.* The low level of the DNA synthesis demonstrated in Fig. 1 shows that during the period of study (150 min) and under the conditions applied, a small fraction of the cells entered DNA synthesis and cell division. Fig. 1 shows further that RNA synthesis was appreciably more increased over the control level than the protein synthesis, in agreement with the hypothesis that F V acts on the level of RNA synthesis [1].

In this study, RNA synthesis was determined by measuring the amount of uridine incorporated into the acid insoluble product of the cells. It is essential to point out that the cells were lyzed by SDS before acid precipitation. This method therefore represents a true measurement of RNA synthesis, because the uridine taken up by the cells but not incorporated into RNA was washed away.

The system was characterized with respect to its requirement for K^+ because of the importance of its intracellular concentration for different metabolic processes. The doubling of RNA synthesis following the addition of K^+ in the absence of NH_4^+ was in agreement with the effect of K^+ in an *E. coli* strain lacking the ability to accumulate K^+ [5], and with *in vitro* studies of DNA polymerase [6]. The optimum external K^+ concentration is about 10 mM, *i.e.* appreciably higher than the level, about 0.1 mM, from which *E. coli* cells in the logarithmic phase are able to accumulate K^+ to the normal intracellular level [5]. Although NH_4^+ can replace K^+ in RNA and protein synthetizing systems *in vitro* [7], it is certainly not the normal *in vivo* activator; its intracellular concentration is maintained normally at 5–10 mM [5]. The suppressing action of NH_4^+ on RNA synthesis in the absence of K^+ is not simply due to a competition with K^+ uptake [8] since the optimum K^+ concentration is shifted to a somewhat lower value in the presence of NH_4^+ (Fig. 2). Part of the effect of NH_4^+ might tentatively be ascribed to an alkalosis produced by passively entering ammonia.

The strong stimulating effect of F V in the absence of K^+ may partly be due to contamination by K^+ of the stimulator, since precipitation of $KClO_4$ is a step in the isolation of the compound. Studies of stimulators of unknown K^+ content in a system which is not used at optimum K^+ concentration are for similar reasons impracticable, and in order to be able to use NH_4^+ salts of reactants, a system with 2 mM K^+ + 50 mM NH_4^+ is considered satisfactory.

The presence of mercaptoethanol in the incubation medium brings the metabolism of the unstimulated (control) system to a lower level, thus increasing the relative response to the stimulator. In the case of F V, mercaptoethanol activates the stimulator, probably by reduction. A similar effect is produced by glutathione, and possibly by cysteamine. The inhibiting action of mercaptoethanol is certainly not associated with oxygen depletion; it probably reflects the depression through some unknown mechanism of nucleic acid synthesis, characteristic of radioprotective thiols [9, 10]. In this context it should be mentioned that mercaptoethanol, although inefficient in mammals, is a good radioprotector in bacteria [11].

The influence of some proteins on the system was studied primarily because of the general use of serum in procedures for lymphocyte stimulation by, *e.g.*, phytohaemagglutinin, in order to get an insight in the possible occurrence of stimulating activities associated with the proteins [12, 13]. The experiment showed that several proteins, but not all, carry such activities, the nature of which is being studied further. In this context, one may mention that different β-lactoglobulin preparations may or may not carry lymphocyte stimulating activity [14].

2. *General characteristics of the E. coli system.* The cells used in the present study were not shocked osmotically in the sense used by HEPPEL [2, 3] since they were not exposed to HEPPEL's "Stage 2", *viz.*, a sudden exposure to low osmotic pressure ("Stage 1"). As with spheroplasts, the cells were kept in a hypertonic medium during the period of study with the difference that treatment with lysozyme and EDTA was omitted. Before the present cell system has more closely been characterized, the cells should be denoted "plasmolyzed". Their protoplasm has been observed to have the same shrunk appearance, with detachment of the membrane from the more rigid cell wall, as observed for *E. coli* cells in 0.4 M NaCl [15]; in fact, NaCl and other solutes may replace sucrose in producing a stimulable cell system. The release of degradative enzymes and phospholipids from the periplasmic space and the membrane, characteristic of Stage 2 of the osmotic shock and of spheroplast preparation, occurs to a limited degree in the Stage 1 exposure to hypertonic solution in the absence of EDTA [16].

The plasmolyzed cells share with HEPPEL's osmotically shocked cells and with spheroplasts the properties of full viability and of increased permeability, as shown above for actinomycin D. If needed, the permeability can probab-

ly be further increased by addition of EDTA to the sucrose solution used for treatment. The low metabolic activity of plasmolyzed cells is probably related to the prolonged lag period of osmotically shocked cells. The prompt increase of the rate of RNA synthesis and other metabolic functions after stimulation of the plasmolyzed cells makes it less likely that they are depleted of precursor pools and essential enzymes. The high optimum K^+ concentration is consistent with the increased permeability or with an injury to active transport mechanisms, as has been observed for amino acids and sugars in osmotically shocked cells. This question, however, requires further study.

On the basis of the demonstrated stimulating activities of a factor from *Phaseolus* seeds and of different proteins, as well as the effects of metabolic inhibitors, we believe that the plasmolyzed bacterial cells described offer a rapid test system useful for the isolation of RNA synthesis stimulating substances from biological material and suitable for studying stimulators and inhibitors of RNA, DNA and protein synthesis.

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DEVELOPMENT OF CELL LINES AFTER EXPOSURE OF CHICKEN EMBRYONIC FIBROBLASTS TO HERPES SIMPLEX VIRUS TYPE 2 AT SUPRAOPTIMAL TEMPERATURE

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Summary. Chicken embryonic fibroblasts were exposed to Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2). Incubation at 40°C for prolonged periods inhibited the production of infectious HSV-2 without preventing most of the cells from degeneration. This procedure, however, resulted in rapid growth of a few cells which survived infection and showed no cytopathic changes and synthesis of infectious virus. Two isolated clones grew into continuous cell lines. Both showed increased resistance to HSV-2 infection, but proved to be susceptible to HSV-1. HSV-specific antigens were found in 5–10% of the cells with indirect immunofluorescent technique. These chicken cell lines afforded an opportunity to study the effect of HSV-2 on cells under non-permissive conditions.

Previous reports have presented sero-epidemiological data associating type 2 herpes infection with carcinoma of the uterine cervix [1–3]. The supposed oncogenicity of HSV-2 was further substantiated by the observation of DUFF and RAPP [4], that transformed and tumorogenic cells developed after exposure of hamster cell cultures to HSV-2 which was inactivated by ultraviolet irradiation.

Theoretically, another possibility of preventing cell destruction and promoting cell transformation is to apply conditions non-permissive for viral replication. Cell-dependent differences in the production of infectious HSV at supraoptimal temperature have been reported [5, 6]; furthermore, the productive replicative cycle of HSV-2 was found more heat sensitive than that of HSV-1 [7, 8]. In this report we describe the development of cell lines following exposure of chicken cells to HSV-2, and incubation at 40°C for prolonged periods.

Materials and methods

Viruses. HSV-1 "HIL" and HSV-2 "LOV" and "BRY" strains were kindly supplied by Professor P. WILDY. HSV-2 No. 45 strain was isolated from a genital eruption in our institute. Virus strains were propagated in HEp-2 cells. Virus was released by ultrasonic disintegration and titrated in HEp-2 cells. Titres were expressed as $TCID_{50}$ according to REED and MUENCH. Virus strains were stored at —70°C until used.

Virus growth. The growth of various strains of HSV-1 and HSV-2 was estimated in secondary chicken fibroblasts and in cell lines established in this study. Cell cultures were grown in tissue culture tubes. Viruses were inoculated onto the cell lines at a multiplicity of 1 and 0.1 $TCID_{50}$/cell, and allowed to adsorb for 1 hr at 37°C. The cultures were rinsed with

phosphate buffered saline (pH 7.2) to remove unattached virus, and overlaid with growth medium (Parker's No. 199 supplemented with 10% calf serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 0.125% $NaHCO_3$). The cell cultures were incubated at 37°C or 40°C and sampled for virus production at various intervals after infection. The virus was extracted from the cells by three cycles of freezing and thawing. Virus yields were expressed as $TCID_{50}$ according to REED and MUENCH.

Virus susceptibility of the cell lines was tested by comparing the titre of virus suspensions in cell lines to that in the control cells, using the plaque titration method.

Cells. Primary chicken embryonic cell cultures were prepared and HEp-2 cells were propagated according to the procedure described by GÉDER *et al.* [9] and GÉDER and VÁCZI [10]. The chicken embryonic fibroblasts were used in the first passage *in vitro*.

Antigens and CF procedures. CF antigens were prepared in PBS by ultrasonic disintegration of a 20% suspension of washed, trypsinized cells grown in tissue culture. After centrifugation at low speed for 20 minutes, the supernatants were used in the tests. HSV-2 CF antigen was prepared from infected HEp-2 cells 48 hours after infection [11].

Immunofluorescence. Inoculation, fixation and staining of cells grown on coverslips was performed as described previously [10].

Immune sera. HSV-2 immune serum 335/XII was kindly supplied by Professor P. WILDY. Preparation of the serum was described by WATSON *et al.* [12]; it had an antibody titre in CF and immunofluorescence of 1/128 as described previously [13].

Virus induction from chicken cell lines. Induction by association with sensitive indicator (HEp-2) cells was performed according to SABIN and KOCH [14, 15].

Results

Growth of HSV-1 and HSV-2 in chicken embryonic fibroblasts at 37°C and 40°C. These experiments were designed to determine the effect of supraoptimal temperature on the synthesis of infectious HSV-1 and HSV-2 in chicken embryonic fibroblasts. M.o.i. 1 $TCID_{50}$/cell. Virus yield of the cells was determined as described under "Materials and methods".

The results show that at 37°C the growth of HSV-1 was more restricted than that of HSV-2, the latter producing titres about 2 exponents higher at 24 hr after infection. However, incubation at 40°C caused total inhibition of HSV-2 replication although the cells degenerated and detached from the glass surface. The supraoptimal temperature reduced and inhibited the growth of HSV-1 but gradually decreasing amounts of infective virus were detected in the cells for a much longer period (Fig. 1).

Development of cell lines. Experiments were made to determine whether there remained surviving cells after HSV-2 infection. Secondary chicken embryonic fibroblasts were inoculated with HSV-2 at a multiplicity of 5 $TCID_{50}$/cell in suspension and adsorbed for 1 hr at 37°C. After repeated washings in growth medium, cells were distributed into culture flasks (2×10^6 cells per flask) and incubated at 40°C; 24 hr later only rounded cells floating in the medium were seen under low power. The flasks were further incubated at 40°C; 6–8 days later, 1–2 small foci of cell growth were detectable on the glass surface in some of the flasks. They grew fast in a piled up manner. Isolated foci were trypsinized and spread over the glass surface to obtain faster growth and sufficient cells for experimental purposes, usually 20 days after infection. They were transferred to 37°C on the 25th day after virus

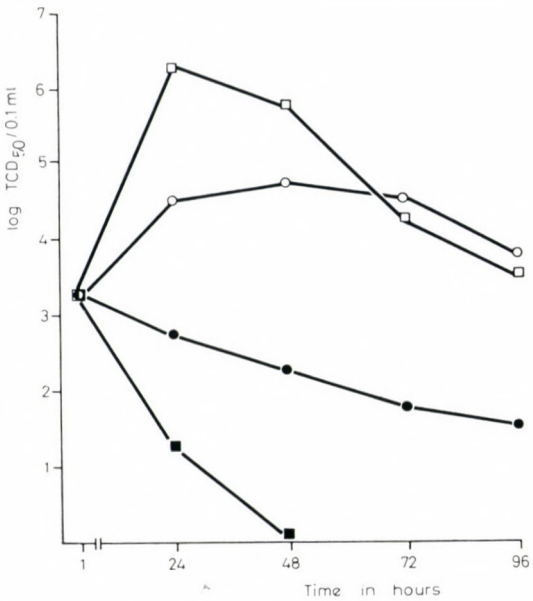

Fig. 1. Growth curves of HSV-1 "HIL" and HSV-2 "LOV" in chicken embryonic fibroblasts at 37°C and 40°C, respectively. ○- - - -○ HSV-1 at 37°C, ●——● HSV-1 at 40°C, □——□ HSV-2 at 37°C, ■——■ HSV-2 at 40°C

inoculation. 12 clones were selected and passaged *in vitro;* 6–10 passages were carried out in the first four months. All the cell lines including uninfected controls began to exhibit reduction in viability at the 4th–7th passage. None of the control cells survived. Ultimately the progeny from 2 of the original clones of HSV-2 infected chicken fibroblasts survived, these cell lines showed a fast growth after the 10th passage, and are now in their 100th tissue culture passage (Fig. 2). The two cell lines described in this paper are Ch_1 and Ch_2 which are fibro-epithelial-like and partially contact-inhibited (Fig. 3).

Assays for infectivity showed that the infectious virus disappeared from the medium 2 days after infection. No infective HSV-2 could be detected from the 20% disintegrates of the cloned cell lines at the 10th tissue culture passage or later.

Growth of HSV-1 and HSV-2 in cell lines Ch_1 and Ch_2. Since the cells were originally infected with HSV-2, growth studies were initiated to determine if HSV-1 and HSV-2 could replicate in these cell lines. They were infected with HSV-1 and HSV-2 at a multiplicity of 0.1 $TCID_{50}$/cell and then the infective virus yield of the cells was determined periodically as described in "Materials and methods". HSV-1 appeared to replicate equally well in both the control and the established cell lines. Growth of HSV-2 was more restricted

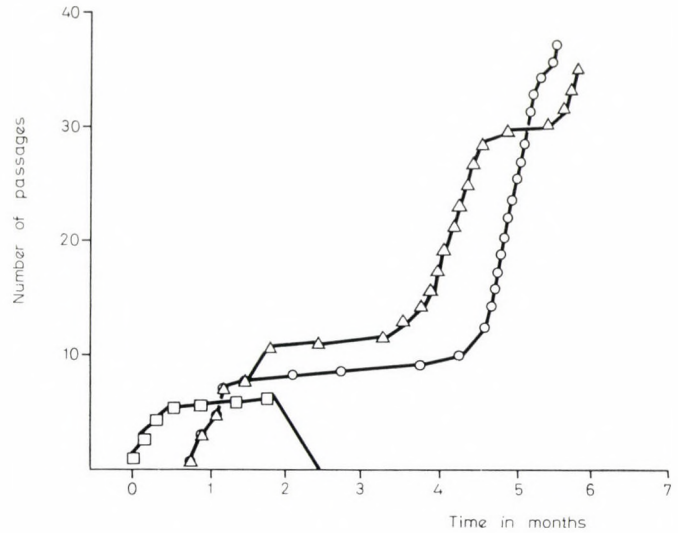

Fig. 2. Development of continuous chicken embryonic cell lines after infection with HSV-2 "LOV". □——□ control uninfected chicken fibroblasts; continuous cell lines: ○——○ Ch_1 △——△ Ch_2

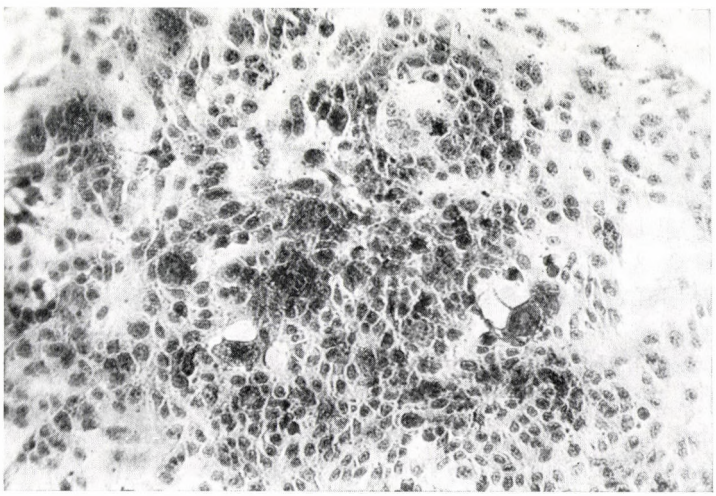

Fig. 3. Continuous chicken embryonic cell line Ch_1. Unstained preparation (×300)

in cells which survived the HSV-2 infection, showing at least 1 exponent difference in top titres in favour of the control cells (Fig. 4).

These results were confirmed in experiments where the susceptibility of cell lines to HSV-2 infection was estimated by comparing the titre of virus suspension obtained by simultaneous titrations in control and our continuous

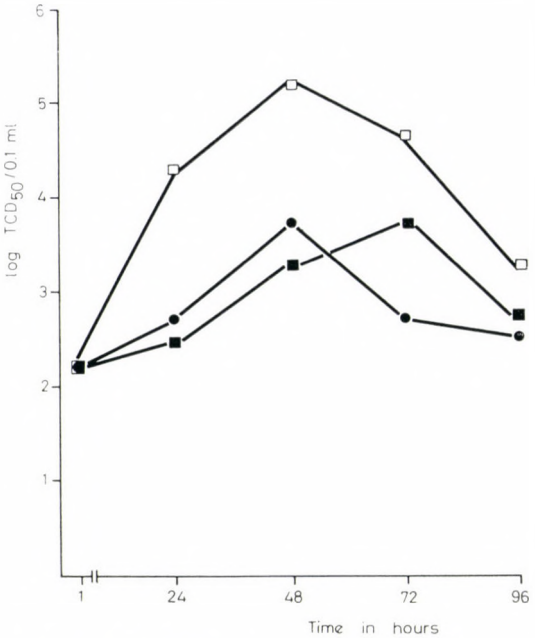

Fig. 4. Growth curves of HSV-2 "LOV" in control uninfected chicken embryonic fibroblasts and in Ch_1 and Ch_2 continuous cell lines. □——□ HSV-2 in control cells, ■——■ in Ch_1 cells, ●——● in Ch_2 cells

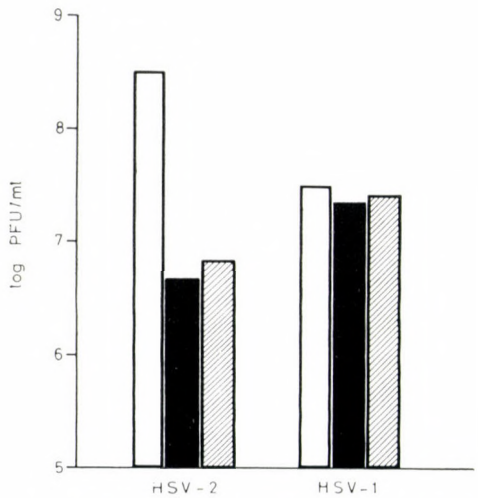

Fig. 5. Titre of HSV-1 "HIL" and HSV-2 "LOV" virus suspensions when determined simultaneously in control chicken cells and continuous lines Ch_1 and Ch_2. □ control cells, ■ Ch_1 cells, ▨ Ch_2 cells

cell lines. When HSV-1 was titrated, equal titres were obtained with the plaque method and normal plaques developed. In each instance, however, the titre of HSV-2 virus suspension in the control cells exceeded by more than one exponent the titre obtained in our continuous cell lines. Slight cytopathic effect was produced by HSV-2 on the resistant cell lines in the form of microscopic plaques, when compared to the macroplaques produced in the more permissive system (Fig. 5).

Search for HSV-specific markers in established cell lines. If these cells are carrying virus genetic material it is possible that they may contain virus-specific markers. When immune serum was used undiluted or in dilution 1 : 2 in indirect immunofluorescent tests, about 5–10% of the cells showed intracytoplasmic fluorescence. Granular surface fluorescence was obtained in unfixed preparations. However, no herpes specific antigens could be detected with immunofluorescence or complement fixation when the immune serum was diluted 1 : 4. None of the control cells showed fluorescence. The cells were also inoculated into freshly hatched chickens to determine any tumorogenic potential. However, the lack of highly inbred chickens for these experiments has made the development of tumours unreliable.

Discussion

Primary chicken embryonic fibroblasts were exposed to HSV-2 and incubated for prolonged periods at 40°C. Following treatment with the virus, the cells showed practically complete degeneration. However, some cells survived the infection and began to grow in "piled up" foci. Twelve such foci were isolated but only two clones resulted in continuous cell lines. They showed increased resistance to productive infection by HSV-2 but were susceptible to HSV-1.

HSV-2 may be oncogenic according to sero-epidemiological data. Herpes infected cells are rapidly destroyed. This situation does not favour cell transformation. The effect is mitigated by inactivation of herpes virus by ultraviolet irradiation or by DMBA [7,12-dimethylbenz(a) anthracene] treatment.

These procedures have the advantage that viral infectivity is destroyed more quickly than the transforming potential [4, 16]. Inoculation of hamster cells with such pretreated virus suspensions results in development of transformed cell lines.

Non-permissive conditions for viral replication may also prevent cell destruction and promote cell transformation. If SV40 tumour cells were infected with HSV-2 and incubated at 40°C, a temporary virus latency developed followed by reappearance of the infectious virus [17]. Incubation of hamster embryonic fibroblasts at 40°C after exposure to HSV-2 resulted in a "transformed" cell line which was tumorogenic in newborn hamsters [18].

The development of our continuous cell lines can be explained on the basis of previous observations, in that supraoptimal temperature delayed the appearance and reduced the quantity of late products of HSV infection, especially in cells of hamster and chicken origin [5, 6, 19]. Such a situation may favour the incorporation of virus genetic material into the cells.

Virus transformed cells are often resistant to superinfection by the same virus. A portion of the HSV-2 genome present in the Ch_1 and Ch_2 cells may be responsible for the observed resistance to HSV-2 infection. The specificity of this inhibition is apparent, as the cells replicate the closely related HSV-1 very well. Immuno-fluorescent studies have also revealed antigens specific for HSV-2 in 5–10% of the cells when immune serum undiluted or in dilution 1 : 2 was used. No infective virus could be induced by association with HEp-2 cells. Anyhow, the development of these cell lines with the help of HSV-2 infection provides a new experimental model for the further investigation of HSV-2 transforming potential in cells under non-permissive conditions.

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HERPES SIMPLEX VIRUS LATENCY IN SV_{40} HAMSTER TUMOUR CELLS

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Summary. Incubation of SV_{40} tumour cell cultures with herpes simplex virus type 2 (HSV-2) at 40°C resulted in rapidly growing cells which survived the infection and lacked both virus cytopathology and synthesis of detectable infectious virus. Following transfer to 37°C infectious HSV-2 reappeared and persistent infection of cells developed without complete destruction of the cell line. There was, however, a delay of at least 8 to 22 days following transfer to 37°C before infectious virus could be detected. HSV-specific immunofluorescent antigens were present in every cell in latency. The reappearance of infectious HSV-2 went parallel with focal cytopathogenic changes. The period between the disappearance and reappearance of infectious virus is considered the latent period. Cultures in this period were less sensitive to superinfection with herpes simplex virus type-2 than control cultures. The reappearance of infectious HSV-2 after prolonged incubation of infected culture at 40°C and 37°C indicates that HSV-2 can remain associated with the cells in a non-infectious form for a prolonged period without being degraded.

Increased resistance to infection by HSV-1 and HSV-2 of SV_{40} hamster tumour cell line was reported previously [1]. The limited response to infection was restricted to the production of infectious virus and virus-specific antigens coded by the progeny viral DNA, but did not extend to the synthesis of "early" antigens synthesized in cells treated with cytosine arabinoside. More transformed cells than infectious virus were capable of producing HSV-specific antigens.

It is shown in the present report that HSV-2 remains associated with these cells in a non-infectious state for various periods when infection was followed by incubation at 40°C.

Materials and methods

Cell cultures. SV_{40} hamster tumour cell line II/d was established from a tumour which developed in a hamster inoculated at birth with SV_{40}, as reported previously [1]. The cell line was in its 80–100th *in vitro* passage when these experiments were performed. Evidence for its transformation by SV_{40} was described previously [1]. Cells were propagated and maintained in Parker's medium No. 199 with 10% or 2% calf serum and 0.125% $NaHCO_3$, respectively.

Viruses. HSV-1 "HIL" and HSV-2 "LOV" strains were obtained from Professor P. Wildy. HSV-2 No. 45 strain was isolated in our Department from a genital eruption. The virus strains were propagated in HEp-2 cells. The virus was released by ultrasonic disintegration and titrated in HEp-2 cells. Titre was expressed as $TCID_{50}$ according to Reed and Muench. Both HSV-2 strains were used in every experiment.

Growth cycle. Growth cycle of virus in control and II/d cells were investigated by measuring the total (intraextracellular) virus yield and expressed as $TCID_{50}$ as described in "Results".

Immune serum. HSV-2 immune serum 335/XII used in immunofluorescent tests was kindly supplied by Prof. P. WILDY. Preparation of immune serum was described by WATSON *et al.* [2]. It had an antibody titre of 1/128, as determined with indirect immunofluorescent test [3].

Immunofluorescent tests. The indirect immunofluorescent method was used [3].

Infectious centre assays. Replicate cultures in the latent phase of infection with HSV-2 were trypsinized and washed once in growth medium. Serial dilutions of suspensions of known number of cells were made and inoculated onto uninfected HEp-2 cultures in Petri dishes growing as monolayers. The cultures were overlaid with Parker's No. 199 medium containing 0.6% DIFCO Noble agar and 0.125% $NaHCO_3$, 3 to 5 days later the cultures were stained with crystal violet and examined for HSV plaques. As a control, the remaining undiluted cell suspension was sonicated and inoculated undiluted onto replicate HEp-2 monolayers.

Results

Growth of HSV-1 and HSV-2 strains in SV_{40} hamster tumour cells at 37°C and 40°C. These experiments were designed to determine the effect of a supraoptimal temperature on the synthesis of infectious virus in SV_{40}-transformed cells. Confluent cell sheets in tissue culture tubes were infected with HSV-1 and HSV-2 at an input of 5 $TCID_{50}$/cell and the virus was allowed to adsorb at 37°C for 1 hr. Cultures were then washed twice with PBS and further incubated at 37°C and 40°C. Three samples of cultures were harvested periodically and assayed for the presence of infectious virus in HEp-2 cells. The virus was extracted from the cells by three cycles of freezing and thawing.

The results show that at 37°C the growth of HSV-2 was more restricted than that of HSV-1 the latter producing titres one log unit higher at 48 hr after infection (Fig. 1). Incubation at 40°C produced total inhibition of replication of HSV-2 although the cell sheet mostly consisted of rounded and degenerated cells detaching from the glass surface. However, some isolated surviving cells remained and began to grow rapidly forming "piled up" foci and soon confluent cell sheets.

The supraoptimal temperature did not prevent but reduced the growth of HSV-1. The surviving cells formed a confluent cell sheet within a short period and continued to yield infectious virus with the appearance of some isolated cytopathogenic foci. This "virus carrier" cell line was maintained during 7 passages without any sign of declination of growth.

Establishment of HSV-2 latency in SV_{40} transformed cells. Experiments were performed to determine if surviving cells of the HSV-2 infected SV_{40}-tumour cell line contained infectious virus or herpes specific antigens.

Tumour cells were inoculated with HSV-2 at a multiplicity of 5 $TCID_{50}$/cell in suspension, and adsorbed at 37°C for 1 hour. After repeated washing in growth medium, cells were distributed into culture flasks (2×10^6 cells per flask) and incubated at 40°C for 24 hr. Most cells became round and floated in the medium, only a few survived and attached to the glass surface. They grew fast, forming about 10 piled up foci by the 3rd day, about 30 by the 5th, and a confluent cell sheet by the 10th to 12th day after infection. They were

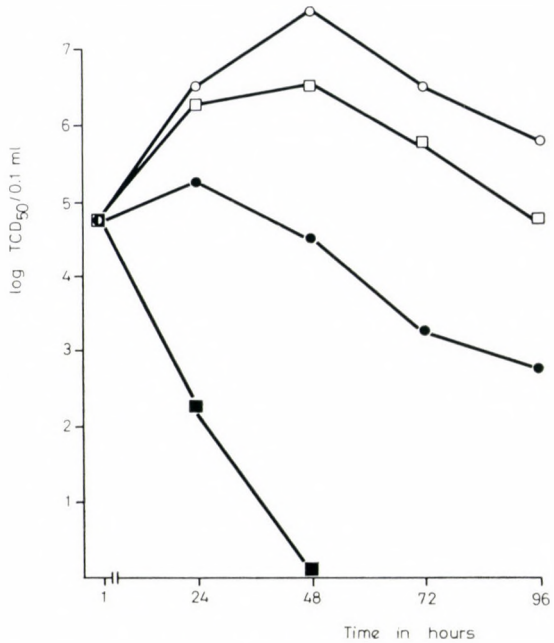

Fig. 1. Growth curves of HSV-1 "HIL" and HSV-2 "LOV" in SV_{40} hamster tumour cells at 37°C and 40°C, respectively. ○——○ HSV-1 at 37°C; ●——● HSV-1 at 40°C; □——□ HSV-2 at 37°C; ■——■ HSV-2 at 40°C.

transferred to 37°C at 16 days after infection. Cell cultures were examined daily for the presence of virus c.p.e. At 4-day intervals, replicate samples were harvested and assayed for the presence of infectious virus on HEp-2 cells. The results of these experiments showed that infectious HSV disappeared from replicate samples at 40°C two days after infection and remained undetectable during the whole period of incubation at 40°C (for 16 days) and for 20 days after transfer of the cells to 37°C. At this time the replicate cultures showed microscopic evidence of virus c.p.e. (Fig. 2). However, the infective virus yield of these cultures remained lower than that of the cultures which had been incubated at 37°C from the beginning of the growth cycle.

Some correlations were found between the length of incubation at 40°C and that of the latent phase which developed after having transferred the cells to 37°C, the latter being longer after more prolonged incubation at 40°C (Table I). However, during the period of viral latency HSV-specific antigens were detected with the indirect immunofluorescent technique in practically every cell. These antigens had predominantly cytoplasmic localization, being often arranged as granular perinuclear formations (Fig. 3).

Growth of HSV types in latently infected SV_{40}-transformed cells. Replicate tumour cell cultures in the latent period as well as uninfected controls were

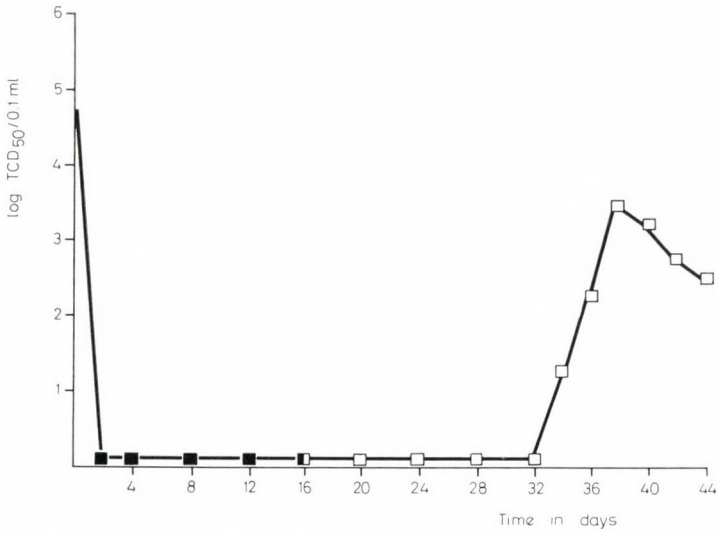

Fig. 2. Course of reappearance of HSV-2 following incubation at 40°C. SV_{40} tumour cells in suspension were inoculated with HSV-2 "LOV" at an input multiplicity of 5 TCD_{50}/cell and allowed to adsorb for 1 hr at 37°C. The cultures were then washed, suspended in growth medium, distributed in glass flasks and incubated at 40°C for 14 days, then transferred to and incubated at 37°C. Replicate samples were harvested periodically and assayed in HEp-2 cells for the presence of infectious virus. ■———■ HSV-2 at 40°C; □———□ HSV-2 at 37°C

Table I

Extension of viral latency and ratio of inducible cells in SV_{40} tumour cell line after infection with HSV-2

Incubation at 40°C, days	Period of latent phase at 37°C, days	Reappearance of infectious HSV-2 (days after infection)	Fraction of cells yielding infectious HSV-2 during latency (infectious centre assays)
6	8	14	.
15	17	32	.
22	23	45	1 : 400
28	22	50	1 : 300

. = Not tested.

tested for their ability to support the synthesis of HSV-1 and 2. The cultures were infected in suspension as mentioned previously and then incubated in tissue culture tubes at 40°C for 30 days. At this time replicate cultures were inoculated as monolayers with either HSV-1 or HSV-2 at a multiplicity of

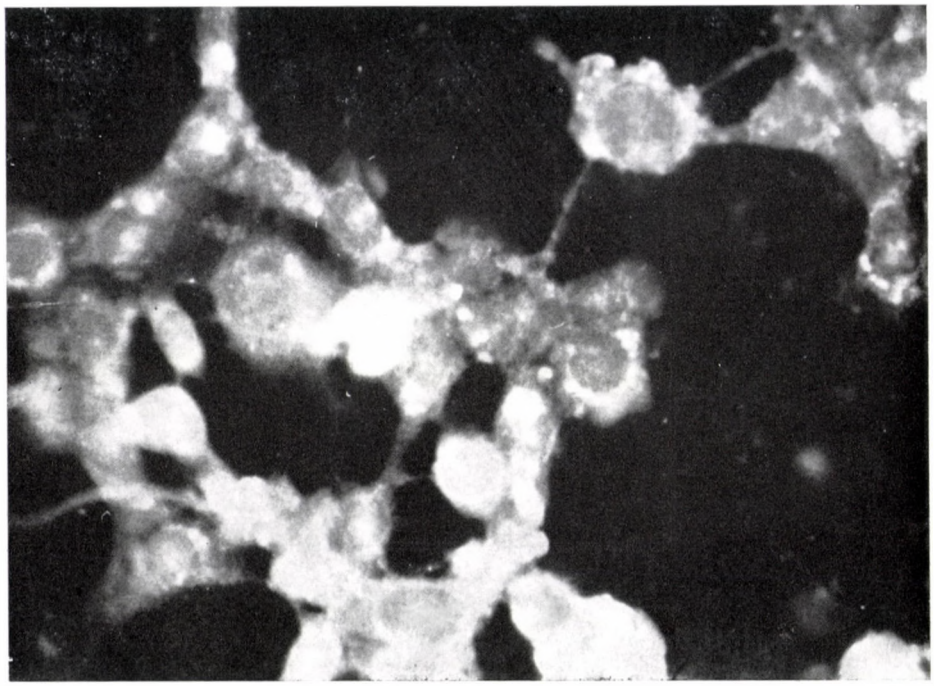

Fig. 3. Intracytoplasmic fluorescence of herpes-specific antigens in SV_{40} tumour cells in the latent phase of infection ($\times 600$)

0.1 $TCID_{50}$/cell. Following adsorption for 1 hr at 37°C the cultures were washed twice, fed with growth medium and incubated at 37°C. Replicate cultures were frozen and thawed three times and tested for infectivity at indicated times after infection. As seen in Fig. 4, HSV-2 growth was more restricted in cells carrying latent HSV-2 and the growth curve of HSV-2 was more "delayed" than that of the control cells. No significant inhibition of the growth of superinfecting HSV-1 was noticed.

Infectious centre assays. Replicate cell cultures were infected with HSV-2 in suspension at a multiplicity of 5 $TCID_{50}$/cell, adsorbed for 1 hr at 37°C, plated and transferred to 40°C for 22 and 28 days. They were incubated at 37°C till the 32nd day after infection, when replicate cultures were harvested for infectious centre assays. Dilutions of virus-exposed cells were added to monolayers of HEp-2 cells. The cultures were examined daily for the presence of foci of HSV-c.p.e., which became apparent between 3 to 5 days after inoculation. The results showed only slight variability in the number of cells synthesizing infectious virus (Table I). The level varied from 1 in 300 to 1 in 400. Infectious virus was not found in the extract of the cells in control assays.

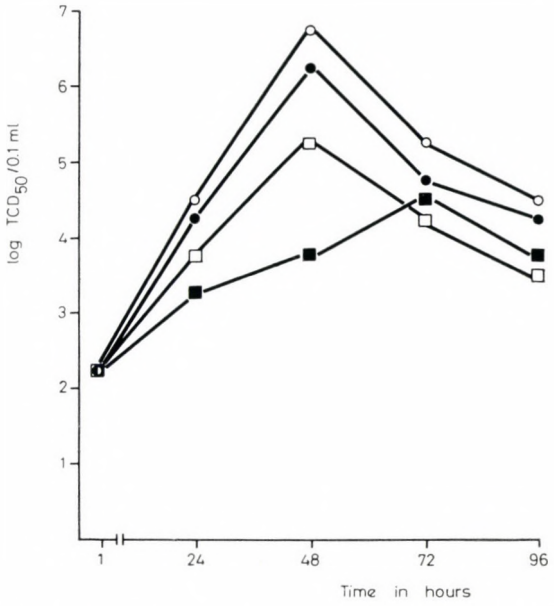

Fig. 4. Susceptibility of latent phase cells to superinfection with 0.1 $TCID_{50}$/cell of either HSV-1 "HIL" or HSV-2 "LOV". Incubation at 37°C. ○———○ HSV-1 in control cells; ●———● HSV-1 in latent phase cells; □-·-·-□ HSV-2 in control cells; ■———■ HSV-2 in latent phase cells

Discussion

O'Neill *et al.* [4] described a human cell culture system in which following infection with HSV-type 2 cell destruction and virus multiplication were prevented by the DNA-inhibitor, cytosine arabinoside, and HSV-2 remained associated with these cells in a non-infectious state for various periods. Before this recent report no attempts at establishing HSV latency *in vitro* have been entirely successful. In all the other systems [5–11] the common feature was a minimal cell destruction, and infectious virus was detected when cells were disrupted.

According to our previous observation [1], the growth of HSV-2 was restricted in SV_{40}-transformed cells which were inducible, had high T-antigen titres and a great tumour potential. Ratcliffe [12] and Longson [13] showed the inhibitory effect of supraoptimal temperatures on the infective yield of HSV-2-infected cells. These findings stimulated us to establish HSV-2 latency in our II/d cell system by incubation at 40°C. This supraoptimal temperature prevented the production of infectious HSV-2 in SV_{40}-transformed hamster cells. Even when cell cultures were transferred to 37°C, there was a delay of at least 1 to 3 weeks before infectious HSV-2 had reappeared. During the period

between the disappearance and reappearance of infectious virus, the HSV-2 was latent. Some cells contained HSV-2 in a non-infectious form, detectable in infectious centre assays. However, herpes-specific antigens localized in the cytoplasm were detected in every cell also in the phase of latency, using the indirect immunofluorescent method. Our observations were similar to those of O'NEILL *et al.* [4] where virus latency was established with cytosine arabinoside.

At this stage of the experiments, the mechanism responsible for the delay in the reappearance of infectious HSV-2 is unclear. It is worth mentioning that STEVENS *et al.* [14] could recover HSV from latently infected trigeminal ganglia of rabbits with recurrent eye infection, only if the ganglia were removed and maintained as organ-cultures for 2–3 weeks.

In our case, the cell-damaging effect of the elevated temperature could be excluded, because (i) the growth of II/d cells was as good at 40°C as at 37°C; (ii) the cells used for virus-yield control in the "superinfection" experiments were also incubated at 40°C prior to virus infection. However, the titre of HSV-2 was 1 log unit higher in these cells than in those which were latently infected. This phenomenon can be interpreted as a sign of highly specific autointerference between the HSV-2 viral genome and viral antigens, in latently infected cells and in the superinfecting HSV-2. If II/d cells were infected with HSV-1, no virus latency developed. The fast-growing cells continuously yielded infectious virus at 40°C.

The effect of elevated temperature on the growth of HSV was studied by different authors. Mainly the late virus antigens are damaged at 40°C, together with a significant or complete block of the production of infective virus [15–17]. Incubation of HSV-infected BS-C-1 cells at 40°C did not reduce the formation of early antigens which are produced also in the presence of cytosine arabinoside, but delayed the appearance and reduced the quantity of the late products of infection [18]. CROUCH and RAPP have confirmed these results finding cell-dependent differences in the production of infectious HSV-2 at 39°C [19]. Hamster embryonic fibroblasts were non-permissive at this temperature. Although c.p.e. had developed, no infectious virus was formed. The production of infectious virus was heat sensitive up to 6 hr after infection. Specific viral antigens were synthesized, but the amount of viral DNA was greatly reduced. However, they do not mention the survival of cells at 39°C after HSV infection.

We have to consider the possibility that some heat-sensitive cell function may be required for the synthesis of HSV-2 DNA or of some critical viral proteins in transformed hamster cells, the restoration of which requires a certain period of incubation at 37°C.

HSV-2 may be oncogenic based mainly on seroepidemiological data and on transformation experiments using UV inactivated virus and hamster embryonic fibroblasts [20]. *In vitro* established HSV latency provides an

experimental model for further investigations of the expression of a possible HSV oncogenic potential in cells under nonpermissive conditions. More detailed studies of this kind of cell–virus relationship are desirable.

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PHAGE TYPING OF SHIGELLA FLEXNERI

I. CLASSIFICATION OF TYPE PHAGES ON THE BASIS OF THEIR SEROLOGICAL PROPERTIES, LYSOGENIZING ABILITY AND LYTIC ACTIVITY

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Summary. A method has been worked out for the phage typing of *Shigella flexneri* strains on the basis of the relation between the type phages. The 19 type phages were classified into 7 serological groups on the basis of neutralization tests with antiphage sera, and into 9 groups, according to immunity developing after lysogenization. The relationship between the phages proved to be almost identical with the two methods. 4606 *S. flexneri* strains were examined by the above phage set and 90 phage types were determined.

The frequency of infections caused by *Shigella flexneri* strains has attracted attention to the need of performing phage typing besides serotyping.

Attempts at working out a phage typing method have been made long ago [1–3]. In Roumania, ISTRATI [4] elaborated a method for dividing the strains of serotype 2a, the most frequent one. After some modifications the phage set consisted of 12 phages.

In Poland, SLOPEK and MULCZYK [5] divided *S. flexneri* strains into 40 phage types. We have introduced both the above mentioned methods [6] and examination of 800 strains showed that according to SLOPEK 43%, and according to ISTRATI 75%, of the strains belonged to two phage types. After comparing the two methods, the two series of phages were partly modified and enlarged with phages isolated in Hungary. Twenty phages were selected for phage typing, and the examined 1474 strains were divided into 124 phage types.

A new phage typing method was then described by the team of SLOPEK [7—9] grouping the phages on the basis of electron microscopic examination.

The purpose of the present study was to modify our phage set, considering the relationship between the phages, and to devise a typing schema more suitable for practical purposes. In doing this we have considered the relationship between the phages by studying their serological properties, their prophage immunity developing after lysogenization by phages and the lytic reactions.

Materials and methods

Phages used for typing schema: SLOPEK's 9 phages (F2, F3, F4, F5, F6, F9, F10, F11, F12), ISTRATI's 5 phages (a, b, c, d, f), 5 Hungarian phages (D2, D8, D14, D17, D19) were used. Typing was performed with the highest dilution of the type phages giving semiconfluent lysis with the propagating strains (RTD).

Strains. Phage sensitivity was determined of 4606 *S. flexneri* strains isolated from patients and carriers in different laboratories in Hungary in the years 1968–1971. The propagating strains have been described in a previous paper [6].

Serological studies. Antiphage sera were prepared with the 19 type phages. Cross-neutralization tests were performed with all sera and all type phages, by the method of ADAMS [10].

Lysogenization. Lysogenizing ability of all 19 type phages on *S. flexneri* strains sensitive to the corresponding phages was tested on agar plates. Fifty strains were lysogenized. Secondary colonies grown at the lytic area were subcultured by streaking them 3 times on agar plates, then examined for phage sensitivity and lysogeny.

Examination of phage relationship on the basis of immunity after lysogenization. Sensitivity of 50 lysogenized strains to a set of 19 phages was examined in order to show the development of immunity against phages lysing the culture before lysogenization.

Results

Serological examinations. The results of neutralization tests with antiphage sera are summarized in Table I. The 19 phages were divided into 7 serogroups according to the neutralizing effect of antiphage sera.

Lysogenization. Lysogenic bacteria are immune to lytic infection, or to lysogenization with the same or closely related phages. This property was used for establishing the relationship between type phages. *S. flexneri* strains were lysogenized with the 19 type phages, and immunity developing after lysogenization was examined. The results are demonstrated in Table II. The phages were arranged according to the serological grouping. On the basis of immunity after lysogenization, 9 groups were distinguished. This subdivision was not identical with the serological grouping though in the case of some phages both yielded the same result.

Grouping of type phages on the basis of their serological, lysogenizing and lytic properties. Table III shows the new phage typing schema. The type phages were divided into 5 groups, the serogroups C, D, E were united into one group.

We determined 90 phage types. Table IV presents the phage type distribution of the examined 4606 *S. flexneri* strains. The most frequent phage types were: 81, 5, 42, 85, 19, 22, 62, 71, 16 and 69.

Discussion

Table I shows that two phages, phage "a" and "F10" belong to serogroup "A" because cross-neutralization developed, though the values of neutralization were low. Serogroup "B" consists of 6 phages (F3, F11, F4, D14, F9, D8). The position of phage "c" is not definite, it was neutralized by

Table I. *Serological classification of S. flexneri type phages*

Serological group	Phages	Titer of neutralization of anti-phage sera																		
		a	F_{10}	F_3	F_{11}	F_4	D_{14}	F_9	D_8	c	F_5	F_6	D_{19}	F_{12}	D_2	D_{17}	F_2	b	f	d
A	a	1000	10								<5									<5
	F_{10}	10	1000																	
B	F_3			1000	10	1000	1000	500	<5				<5							
	F_{11}			100	100	10	10	10	10	100			<5							
	F_4			100	100	1000	100	10	100	10			<5							
	D_{14}			100	100	100	1000	10	100	100			<5							
	F_9			100	10	10	100	500	10	10			<5							
	D_8			<5	<5	10	100	100	100	<5			<5							
C	c			<5	10	10	10	100	<5	1000			<5							
D	F_5								<5	1000	1000	1000	1000							
	F_6							<5		1000	1000	1000	1000							
	D_{19}								<5	1000	1000	100	1000			<5	<5			
E	F_{12}										1000			1000						
F	D_2													10	1000	1000				
	D_{17}		<5											10	1000	1000	100			
	F_2															100	1000			
G	b																	1000	10	10
	f	<5																100	10000	10000
	d																	100	10000	10000

Table II

Classification of S. flexneri type phages on the basis of prophage immunity after lysogenization

Group	Phages used for lysogenization	Type phages																		
		a	F_{10}	F_3	F_{11}	F_4	D_{14}	F_9	D_8	c	F_5	F_6	D_{19}	F_{12}	D_2	D_{17}	F_2	b	f	d
1	a	1	1							2	2	2								
	F_{10}	1	1			2														
2	F_3			1	1	1	1	1												
	F_{11}			1	1	1	1	1												
	F_4			1	1	1	1	1												
	D_{14}			1	1	1	1	1												
	F_9			1	1	1	1	1												
3	D_8								1											
4	c								1	1	1	1	1	2	1	1		2		
	F_5								1	1	1	1	1	2	1	1		2		
	F_6								1		1	1	1							
5	D_{19}						1						1							
6	F_{12}													1						
7	D_2														1	1		1		
	D_{17}														1	1				
8	F_2																1			
9	b																	1	1	1
	f																	1	1	1
	d																	1	1	1

1 immunity to every lysogenized strain
2 no immunity to every lysogenized strain

antiphage sera F11, F4, D14 and F9, thus in this respect it belongs to group "B", but it is related with group "D" too, because antiphage sera produced with phage "c" neutralized phages D19, F5 and F6. Therefore, phage "c" forms group "C" alone and means a link between groups "B" and "D". Group "E" consists of phage F12. Though phage F12 was neutralized by antiphage sera F5, phage F5 was not neutralized by antiphage sera F12 — thus it forms an independent serological group. Phages D2, D17 and F2 belong to group

"F". Weak neutralization developed to phage F12. Group "G" consists of 3 serologically homologous phages (b, f, d).

Table II shows the immunity developing after lysogenization. Phages "a" and "F10" belong to one group ("1"), in agreement with serological classification ("A"). Lysogenizing with phage "a" or "F10", the strain loses its sensitivity to both phages. Group "2" consists of 5 phages (F3, F11, F4, D14, F9). Phage D8 forms an independent group ("3"); lysogenizing with phage D8 induces immunity only to the homologous phage.

Group "4" consists of 3 phages: phage "c", F6 and F5. Lysogenization with these phages provides immunity to phages D8 and D19 too, but it is not a cross immunity. D19 is the only phage in group "5".

Table II shows that immunity has not developed to phage "c" after lysogenization with phage F6. The explanation is that the original non-lysogenic strain was not sensitive to phage "c" either.

"F12" is the only phage in group "6", because lysogenization with F12 did not result in cross immunity to any other phage, not even to phage F5, though after lysogenization with phage F5 one out of the two strains induced immunity to phage "F12" too.

Phages D2 and D17 belong to group "7". Cross-immunity has been revealed in lysogenization with these phages. Phage F2 belongs to group "8" as this phage was not related to any other phage on the basis of immunity developing after lysogenization. Group "9" consists of 3 phages: "b", "f" and "d" according to the cross-immunity obtained.

Comparing the serological and immunity groups: serogroup "A" is identical with the immunity group "1", serogroup "B" includes the phages of group "2" and "3". Phages D2 and D17 are related (group "F" and "7"). Phage F2 belongs serologically to group "F", related to D2 and D17, but on the basis of immunity it was separated in group "8". Phages b, f, d are related according to both classifications, and belong to groups "G" and "9".

The phage typing schema was devised on the basis of the above groupings, taking into consideration the lytic patterns (Table III). The schema consists of 90 phage types. The connection between the serotypes and phage types of *S. flexneri* strains, and their use in epidemiological practice will be reported in another paper.

Table III

Phage typing schema for S. flexneri

Phage type	Groups of type phages																		
	I		II						III					IV			V		
	a	F_{10}	F_3	F_{11}	F_4	D_{14}	F_9	D_8	c	F_5	F_6	D_{19}	F_{12}	D_2	D_{17}	F_2	b	f	d
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	−
8	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	+
10	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−
11	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	−
12	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−
13	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	−
14	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	+	+	−
16	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	+	−	−
17	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	−	−
18	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−
19	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−
20	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	+	+	+
21	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	+	−	+
22	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	+	−	−

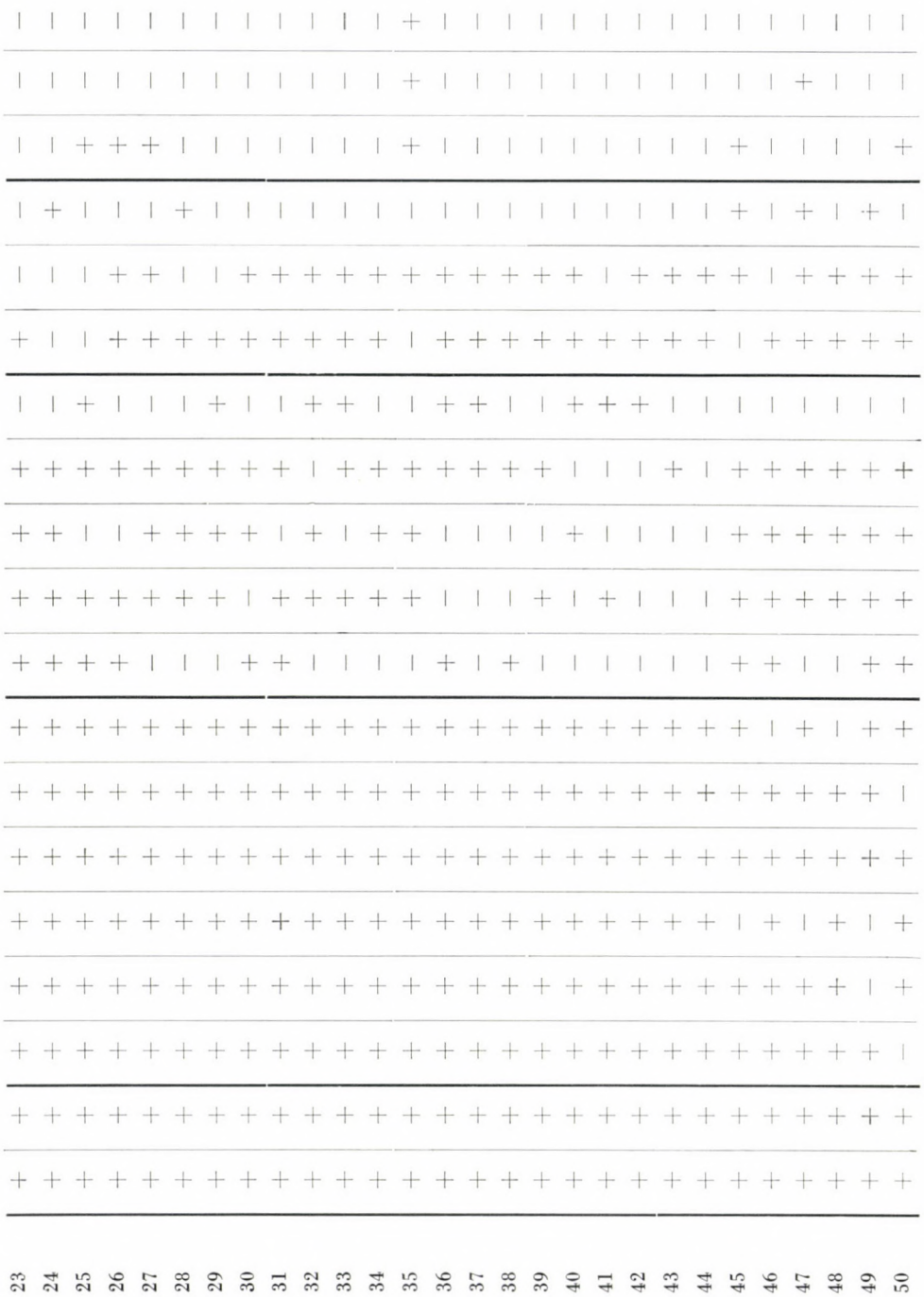
23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50

Table III (continued)

Phage type	Groups of type phages																	
	I		II						III					IV			V	
	a	F_{10}	F_3	F_{11}	F_4	D_{14}	F_9	D_8	c	F_5	F_6	D_{19}	F_{12}	D_2	D_{17}	F_2	b	f	d
51	+	+	−	+	+	+	−	+	−	+	−	+	−	+	−	+	−	−	−
52	+	+	−	−	−	+	−	+	+	+	+	+	−	+	+	+	+	−	−
53	+	+	−	−	−	+	−	+	+	+	+	+	−	+	−	+	+	−	−
54	+	+	−	−	−	+	−	+	+	+	+	+	−	+	−	−	+	−	−
55	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−
56	+	+	−	−	−	−	−	+	+	+	+	+	−	+	+	+	+	−	−
57	+	+	−	−	−	−	−	+	+	+	+	+	−	+	+	+	−	−	−
58	+	+	−	−	−	−	−	+	−	+	+	+	+	+	+	−	+	−	−
59	+	+	−	−	−	−	−	+	+	+	+	+	−	−	−	+	+	−	−
60	+	+	−	−	−	−	−	+	+	+	+	−	+	−	−	−	−	−	−
61	+	+	−	−	−	−	−	+	−	+	−	+	+	+	+	+	+	−	−
62	+	+	−	−	−	−	−	+	+	+	+	−	−	−	−	+	−	−	−
63	+	+	−	−	−	−	−	+	−	+	+	+	−	−	−	−	−	−	−
64	+	+	−	−	−	−	−	+	−	+	−	−	+	+	+	−	−	−	−
65	+	+	−	−	−	−	−	+	−	+	−	−	+	−	−	+	+	−	−
66	+	+	−	−	−	−	−	+	−	+	−	+	−	+	−	−	−	−	−
67	+	+	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−
68	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
69	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−
70	+	−	+	+	+	+	+	+	+	+	+	+	−	+	−	+	−	−	−
71	+	−	+	+	+	+	+	−	+	+	+	+	−	+	+	−	−	−	−
72	+	−	−	−	−	+	−	+	+	+	+	+	−	+	+	+	−	−	−
73	+	−	−	−	−	−	−	+	+	+	+	+	−	+	+	+	−	−	−

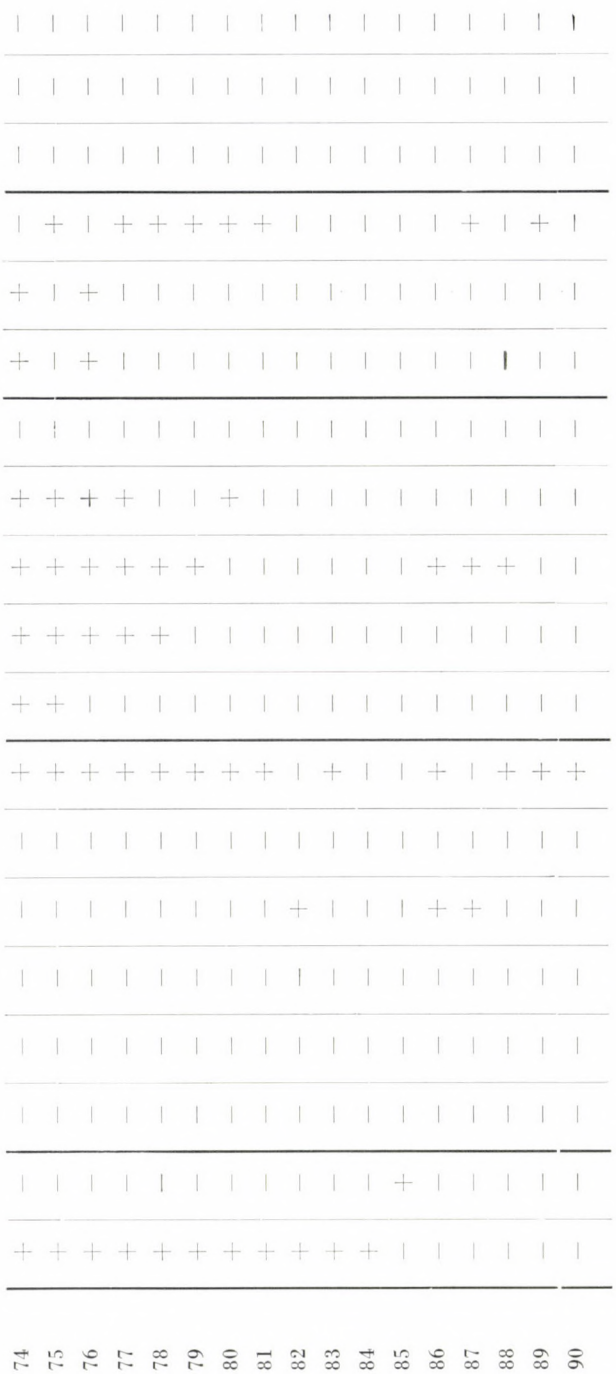
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Table IV

Phage type distribution in percentage of S. flexneri strains in the period 1968 to 1971

Phage type	Year				Total
	1968	1969	1970	1971	
1	—	—	0.4	—	0.1
2	0.2	0.6	0.6	0.8	0.5
3	—	—	2.1	1.6	0.8
4	0.3	0.1	—	1.3	0.4
5	7.9	19.1	1.7	9.3	10.1
6	0.1	0.6	—	—	0.2
7	0.2	0.1	0.6	0.1	0.2
9	0.8	0.1	0.7	—	0.4
10	2.3	0.1	1.1	1.0	1.1
12	—	—	—	0.6	0.1
13	0.5	0.9	0.2	0.1	0.4
14	0.4	—	0.4	1.3	0.5
15	—	—	0.8	0.1	0.1
16	6.0	0.2	3.1	2.7	3.0
17	0.5	0.3	—	0.1	0.2
18	—	—	—	1.8	0.5
19	0.2	5.8	23.6	8.1	8.1
20	2.4	5.2	0.2	—	2.1
21	1.1	—	—	—	0.3
22	4.7	8.4	6.2	—	4.7
23	3.0	2.1	0.1	0.2	1.6
24	0.2	—	—	0.1	0.1
25	0.2	—	—	0.3	0.2
26	—	—	—	3.0	0.8
27	0.2	—	—	0.3	0.3
28	0.5	0.1	—	0.1	0.2
29	0.8	—	—	—	0.2
30	0.1	—	—	—	0.1
31	—	—	—	3.6	0.9
32	0.4	4.2	—	—	1.2
33	—	—	3.3	0.1	0.6
34	0.2	—	0.1	—	0.1
36	—	—	—	0.3	0.1
37	0.4	0.1	4.8	0.2	1.1
38	0.1	0.3	0.7	0.8	0.4
39	—	—	0.9	1.0	0.4

Table IV (continued)

Phage type	Year				Total
	1968	1969	1970	1971	
40	2.2	0.6	—	—	0.8
41	1.1	—	0.1	—	0.3
42	22.0	8.3	5.2	0.6	9.4
43	—	—	4.0	0.7	0.8
44	0.7	—	0.9	—	0.4
45	0.2	—	—	—	0.1
46	—	1.3	2.1	—	0.7
47	0.5	—	—	—	0.1
48	0.2	—	1.2	—	0.3
49	0.1	0.2	—	—	0.1
50	0.2	—	—	—	0.1
51	—	—	0.1	—	0.1
52	4.4	0.1	0.1	—	1.2
53	0.2	—	0.1	—	0.1
54	0.1	0.1	0.8	—	0.2
55	—	—	—	0.5	0.1
56	0.1	—	0.8	0.3	0.4
57	—	—	0.8	0.2	0.2
58	0.1	—	—	0.2	0.2
59	1.8	1.1	—	—	0.8
60	—	0.6	0.5	—	0.2
61	0.2	—	—	0.5	0.2
62	6.1	8.5	2.8	—	4.5
63	—	—	—	0.2	0.1
64	—	—	—	0.9	0.2
66	—	—	—	0.1	0.1
67	1.0	1.6	2.8	0.2	1.2
68	1.4	0.2	0.2	0.1	0.5
69	—	—	—	6.4	1.7
70	—	—	—	0.4	0.1
71	—	—	—	11.8	3.2
72	0.1	—	0.1	0.2	0.1
73	0.2	0.2	2.0	1.8	0.9
75	—	—	—	0.2	0.1
76	—	—	—	1.1	0.3
78	—	—	0.2	3.8	1.1
80	0.1	—	—	—	0.1
81	4.9	14.3	15.8	15.0	12.2

Table IV (continued)

Phage type	Year				Total
	1968	1969	1970	1971	
82	—	0.1	—	—	0.1
83	—	—	0.4	1.7	0.5
84	0.2	0.8	1.2	1.9	1.0
85	9.7	8.4	4.0	9.8	8.3
86	0.4	—	—	—	0.1
87	1.8	—	—	—	0.5
89	1.6	0.2	0.2	0.9	0.8
Degraded	—	0.6	0.8	0.7	0.5
N. t.	4.7	4.7	1.2	0.9	3.0
Total No. of strains	1286	1233	843	1244	4606

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PHAGE TYPING OF SHIGELLA FLEXNERI

II. EPIDEMIOLOGICAL OBSERVATIONS

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Summary. Phage types of 4606 *Shigella flexneri* strains were examined in the years 1968–1971. Of the examined strains, 78% were isolated from sporadic cases, family outbreaks and from control examinations, 22% originated from local and institutional outbreaks. There was no definite correlation between serotypes and phage types. The most frequent phage types were: 81, 5, 42, 85, 19, 22, 62, 71, 16 and 69. The dominant phage types isolated from local outbreaks were: 5, 19, 22, 71, 73 and 85. The dominant phage types originating from institutional outbreaks were: 5, 13, 14, 16, 19, 20, 21, 22, 23, 28, 31, 32, 41, 42, 54, 59, 71, 76, 78· 81, 83, 85 and 89.

Phage typing proved to be suitable in epidemiological practice for subdividing the serotypes, for tracing the infection routes and for distinguishing between cases from different foci.

A *Shigella flexneri* phage typing method has been described in the previous paper [1]. The purpose of the present paper is to discuss the use of the method in practice.

Materials and methods

Materials and methods have been described previously [1].

Results

Distribution of phage types. According to the data of the last 4 years, however, *S. flexneri* strains belonged in 30–40% to serotype 3a in Hungary, therefore it was necessary to introduce phage typing for subdividing the serotypes for epidemiological purposes.

We examined 4606 *S. flexneri* strains in the years 1968–1971. Table I shows the distribution of strains according to serotypes. The most frequent serotype was 3a (39.1%), the next in order was serotype 2a (21.2%).

Fig. 1 and Table II demonstrate the distribution of serotypes by phage typing. A total of 1800 strains, belonging to serotype 3a, were divided into 57 phage types. Three phage types were predominant: 42, 5 and 19 occurring in 23.0%, 22.5% and 20.1%, respectively. Further 6 phage types (37, 43, 31, 67, 26 and 40) occurred in 2.5–1.9%. The other 48 phage types occurred in less than 1.9%.

Table I

Distribution of S. flexneri strains according to serotype, in percentage (1968—1971)

Serotype	Year				Total
	1968	1969	1970	1971	
1a	0.7	0.9	0.6	0.2	0.6
1b	12.7	7.5	6.0	3.1	7.5
2a	23.4	19.9	17.0	23.2	21.2
2b	0.5	0.1	0.2	0.1	0.3
3a	39.0	39.8	47.8	32.7	39.1
3b	—	—	0.1	0.9	0.3
3c	—	—	0.1	0.3	0.1
4a	8.9	14.8	14.7	21.6	14.9
4b	0.3	0.2	3.0	0.4	0.8
5	—	—	0.1	0.1	0.1
6	9.6	8.6	4.7	11.0	8.8
var. X	4.5	6.5	5.6	5.9	5.6
var. Y	0.2	1.3	0.1	0.4	0.5
1b—3b	0.2	0.4	—	0.1	0.2
No. of strains examined	1286	1233	843	1244	4606

The examined 977 strains of serotype 2a were distributed into 51 phage types. The incidence of phage types 22, 71 and 16 was higher than 10% (20.6%, 15.0% and 12.7% respectively), while 3 phage types (20, 69 and 23) occurred in 5–10%; 30% of the strains belonged to 48 other phage types.

Out of 689 strains of serotype 4a, 71.8% belonged to phage type 81. The frequency of phage type 78 was 6.9%, that of phage type 89 was 4.6%; 26 other phage types amounted to 16.7% of the strains.

Serotype 6 strains belonged to phage type 85 in 87.9%, while 12.1% of the strains fell into 18 other phage types. Out of 344 strains of serotype 1b 39.5% belonged to phage type 62. The occurrence of phage types 52, 73 and 59 was between 16.2 and 6.6%. The frequency of 29 other phage types was less than 4%.

The other serotypes and variants were not as frequent as the above mentioned ones, but all of them could be subdivided by means of phage typing.

Fig. 2 and Table III show the correlation between the ten most frequent phage types and serotypes. Phage type 81 was the most frequent — to this type mainly serotype 4a and 4b belonged, and 8 other serotypes occurred among the examined strains. The majority of phage types 19, 5 and 42 was

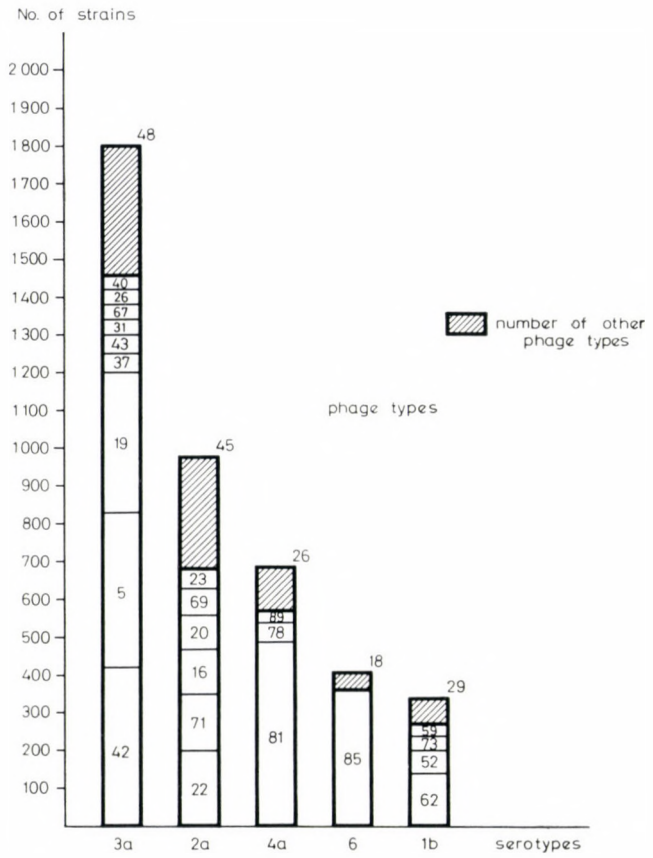

Fig. 1. Correlation of frequent serotypes and phage types of *S. flexneri* strains in Hungary (1968–1971)

of serotype 3a, but all of them involved 4, 7, and 5 other serotypes. Serotype 6 was dominant in phage type 85, whereas 5 other serotypes were found. 2a was the dominant serotype of phage type 22, 16 and 71 — but only phage type 71 was serologically homogeneous, 4 and 6 other serotypes belonged to phage type 22 and 16, respectively.

Fig. 3 represents the distribution according to counties of the frequent phage and serotypes of *S. flexneri* strains, examined in our laboratory in 1971.

Strains of serotype 3a belonged to the following phage types: in Budapest 5; in Pest county 5 and 19; in Győr-Sopron, Veszprém and Zala counties 5; in Nógrád and Csongrád counties 19 and 26. Strains of serotype 2a were in Budapest of phage type 69 and 71; in Pest, Veszprém and Tolna counties 71; in Nógrád and Szolnok counties 16; in Békés county 69.

Table II

Distribution of S. flexneri serotypes according to phage type (1968—1971)

Total no. of strains, 4606

Serotype	No. of strains	Frequent phage types	No. of strains	No. of rare phage types	Rare phage types
1a	28	62	10	10	1, 10, 16, 17, 22, 67, 72, 75, 81,
		59	4		89, Nt
1b	344	62	136	29	5, 7, 9, 10, 12, 16, 17, 20, 22, 23, 27,
		52	56		28, 41, 42, 49, 53, 56, 60, 61, 67,
		73	34		68, 70, 72, 75, 76, 81, 84, 85, 89,
		59	23		Degr., Nt.
2a	977	22	202	45	1, 2, 3, 4, 5, 6, 7, 9, 10, 14, 15, 17,
		71	147		18, 19, 21, 24, 25, 28, 29, 34, 38,
		16	125		40, 41, 42, 44, 45, 46, 47, 48, 50,
		20	84		53, 54, 56, 58, 59, 60, 62, 67, 68
		69	72		73, 76, 81, 84, 85, 87, Degr., Nt.
		23	53		
2b	12	68	5	6	2, 14, 16, 20, 22, 31
3a	1800	42	415	48	2, 4, 7, 9, 10, 13, 14, 16, 18, 19, 20,
		5	407		21, 22, 23, 25, 27, 28, 29, 30, 32,
		19	363		33, 34, 36, 38, 39, 41, 44, 45, 47,
		37	47		50, 52, 54, 57, 58, 59, 60, 61, 62,
		43	42		63, 64, 68, 69, 81, 83, 84, 85, 86,
		31	39		89, Degr., Nt.
		67	38		
		26	37		
		40	35		
3b	12	14	4	5	3, 5, 16, 18, 55
3c	5	—	—	5	12, 19, 55, 60, 69
4a	689	81	495	26	2, 5, 6, 7, 10, 15, 28, 31, 37, 42, 51,
		78	48		59, 61, 62, 63, 67, 68, 69, 73, 80,
		89	32		82, 83, 84, 85, 86, 87, Degr., Nt.
4b	37	81	29	5	18, 19, 62, 72, 78
5	2	—	—	2	16, 84
6	407	85	358	18	5, 9, 10, 17, 20, 21, 23, 27, 41, 42, 46,
					62, 70, 73, 81, 83, 84, 89, Degr., Nt.

Table II (continued)

Serotype	No. of strains	Frequent phage types	No. of strains	No. of rare phage types	Rare phage types
var. X	259	32	49	32	2, 4, 7, 9, 10, 12, 13, 17, 18, 19, 20,
		5	31		23, 24, 28, 29, 31, 37, 38, 42, 44,
		3	30		55, 60, 61, 62, 63, 66, 67, 69, 81,
		84	21		83, 85, 89, Degr., Nt.
var. Y	25	—	—	12	2, 5, 10, 14, 23, 28, 32, 62, 68, 81, 84, 85, Nt.
1b—3b	9	—	—	6	6, 14, 20, 23, 49, 81, Nt.

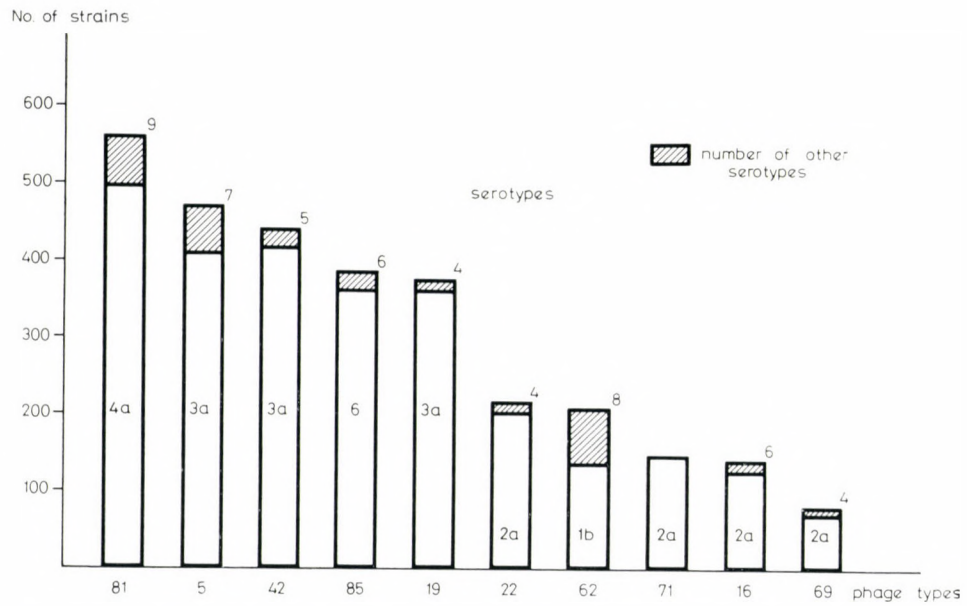

Fig. 2. Correlation of frequent phage types and serotypes of *S. flexneri* strains in Hungary (1968–1971)

Strains of serotype 4a were distributed as follows: phage type 81 in Pest, Nógrád, Csongrád counties and phage type 83 in Szolnok county. Strains of serotype 6 belonged to phage type 85 in both Csongrád and Veszprém counties.

Phage typing of S. flexneri strains originating from dysentery outbreaks. Phage typing was carried out in the case of 54 outbreaks caused by *S. flexneri* strains in the years 1968–1971. Seven local and 47 institutional outbreaks were studied, the number of patients was 553 in the former, and 1041 in the

Table III

Distribution of frequent S. flexneri phage types according to serotype (1968—1971)

No.	Phage type	No. of strains	Dominant serotype	No. of strains of dominant serotype	Other serotypes
1	81	560	4a	495	1a, 1b, 2a, 3a, 4b, 6, var. X, var. Y 1b—3b
2	5	467	3a	407	1b, 2a, 3b, 4a, 6, var. X, var. Y
3	42	437	3a	415	1b, 2a, 4a, 6, var. X
4	85	384	6	358	1b, 2a, 3a, 4a, var. X, var. Y
5	19	374	3a	363	2a, 3c, 4b, var. X
6	22	216	2a	202	1a, 1b, 2b, 3a
7	62	207	1b	136	1a, 2a, 3a, 4a, 4b, 6, var. X, var. Y
8	71	147	2a	147	—
9	16	140	2a	125	1a, 1b, 2b, 3a, 3b, 5
10	69	79	2a	72	3a, 3c, 4a, var. X

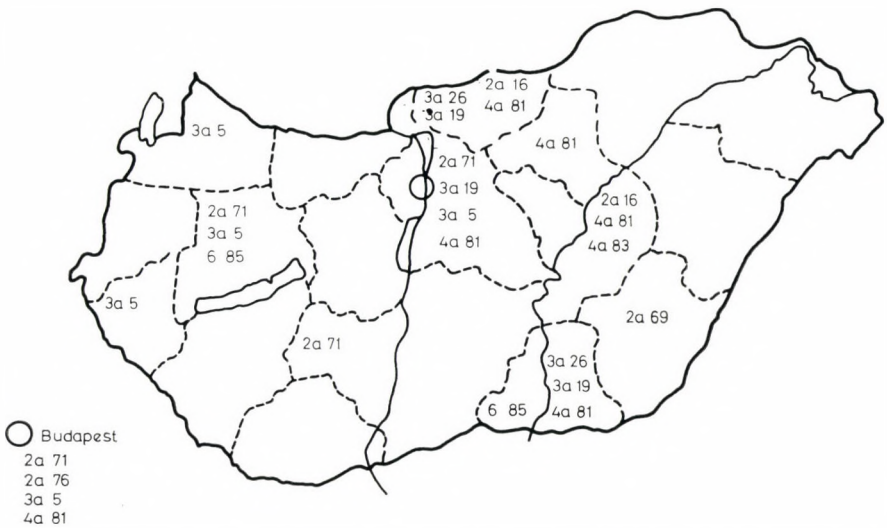

Fig. 3. Serotype and phage type distribution of the examined *S. flexneri* strains in Hungary in 1971

latter, the number of examined strains was 231 and 763, respectively (Tables IV–VIII).

Polluted water was the vehicle of infection in 4 cases and food in 2 cases; the others were contact cases. The number of predominant phage types was 24.

Table IV

S. flexneri serotypes and phage types originating from dysentery outbreaks (1970—1971)

Epidemic serotype	No. of			Dominant phage type	Other phage type
	examined outbreaks	patients	examined strains		
1b	1	10	7	73	22
2a	2	30	23	22	16
		86	77	71	18
3a	3	12	12	19	—
		20	30	5	—
		20	23	19	—
6	1	375	61	85	83 Degr. Nt.
Total 1b, 2a, 3a, 6	7	553	233	5, 19, 22, 71, 73, 85	16, 18, 22, 83, Degr., Nt.

Table V

S. flexneri serotypes and phage types originating from dysentery outbreaks in institutions (1968)

Epidemic serotype	No. of examined outbreaks	No. of patients	No. of examined strains	Dominant phage type	Other phage type
1b	1	10	9	59 and 62	—
2a	4	20	13	22	25
		10	8	21 and 23	20
		10	5	22	4
		20	14	16	—
3a	3	20	15	42	—
		10	6	41	—
		10	5	42	—
3a 6	1	50	41	5 85	40
6	1	20	17	85	
Total 1b, 2a, 3a, 6	10	180	133	5, 16, 21, 22, 23, 41, 42, 59, 62, 85	4, 20, 25, 40

Table VI

S. flexneri serotypes and phage types originating from dysentery outbreaks in institutions (1969)

Epidemic serotype	No. of examined outbreaks	No. of patients	No. of examined strains	Dominant phage type	Other phage type
2a	1	10	11	22	—
2a	2	10	7	20	—
3a				42	—
		30	28	20	—
				5	—
3a	7	6	6	42	—
		12	11	19	5, 40
		7	7	5	—
		5	4	5	19
		18	16	5	42
		10	9	5	—
		40	37	5	—
3a	1	22	19	5	40
4a				81	
3a	1	21	18	5	13
6				85	
4a	4	12	11	81	—
		20	19	81	—
		15	15	81	—
		8	9	81	Nt
4a	1	79	78	81	37
6				85	
6	1	5	5	85	—
var. X	1	40	41	32	17
Total 2a, 3a, 4a, 6, var. X	19	370	351	5, 19, 20, 22, 32, 42, 81, 85	5, 13, 17, 19, 37, 40, 42, Nt

In local outbreaks, predominant phage types were: 19, 22, and 73 in 1970; 19 and two others in 1971.

Frequent phage types were in institutional outbreaks: 16 (in 1968, 1970, 1971), 22 (in 1968, 1969), and 19 (in 1968, 1969, 1970); 42 and 85 were also

Table VII

S. flexneri serotypes and phage types originating from dysentery outbreaks in institutions (1970)

Epidemic serotype	No. of examined outbreaks	No. of patients	No. of examined strains	Dominant phage type	Other phage type
2a	2	20	9	16	—
		9	9	54	16
3a	4	6	6	19	—
		16	16	19	—
				42	
		14	11	37	43
		6	6	19	—
4a	1	12	11	89	—
var. X	1	22	12	41	—
Total 2a, 3a, 4a, var. X	8	105	80	16, 19, 37, 41, 42, 54, 89	16, 43

Table VIII

S. flexneri serotypes and phage types originating from dysentery outbreaks in institutions (1971)

Epidemic serotype	No. of examined outbreaks	No. of patients	No. of examined strains	Dominant phage type	Other phage type
2a	4	7	20	16	—
				14	
		143	18	16	73
		63	23	71	—
		4	7	76	—
3a	2	12	13	5	—
		5	5	31	—
4a	3	17	22	81	—
		73	45	78	—
		47	27	81	—
				83	
6	1	15	22	85	—
Total 2a, 3a, 4a, 6	10	386	202	5, 14, 16, 31, 71, 76, 78, 81, 83, 85	73

dominant for 2–3 years; the predominance of other phage types alternated yearly.

Phage typings of S. flexneri strains originating from the German Democratic Republic and Bulgaria. We received 50 *S. flexneri* strains isolated in the G.D.R. in 1970: 48 strains were of serotype 3a and 2 strains of serotype 5. Strains of serotype 5 belonged to phage type 27 — a phage type which in Hungary occurred only in 0.3% during the four years of examination. Among the strains of serotype 3a, 23 strains originated from sporadic cases and 25 strains from group-diseases. Strains isolated from sporadic cases belonged to phage types 27, 33, 37, 42 and 44; the phage types of the strains causing group-diseases were 25, 27, 33, 37 and 43.

We determined phage types of 11 strains isolated from 3 epidemics in Bulgaria in 1971. The strains were of serotype 2a and of phage types 69 and 73.

Discussion

The strains examined in our laboratory yielded only representative data concerning the causative agents of dysentery in Hungary. In the years 1968 to 1970, 36 743 cases of dysentery were reported in Hungary, and in 48.4% *S. flexneri* was responsible for them. During these 3 years we examined 3362 strains, *i.e.* 19% of the reported cases.

In our material strains of serotype 3a were predominant, their occurrence was 39.0–47.8%. They were followed by serotype 2a (17.0–23.4%). The frequency of serotype 4a was fluctuating (8.9–21.6%). The occurrence of serotype 6 was 8.8% on the average, fluctuating between 4.7–11.0%. Serotype 1b and var. X were also frequent (7.5% and 5.6%, respectively), the other serotypes varied between 0.1–0.8%.

All serotypes could be subdivided by phage typing, and the usefulness of this method has been proved for epidemiological purposes.

No close correlation could be observed between serotypes and phage types. As to frequent phage types, for example, 560 strains belonged to phage type 81; out of these 88.3% were of serotype 4a; 11.7% were distributed between other serotypes. Among serotypes 2b, 3b, 3a we have failed to find phage type 81.

Phage type 85 belonged in 93.2% to serotype 6, but it was detected in serotypes 1b, 2a, 2b, 4a as well as in var. X and Y.

Phage type 71 belonged exclusively to serotype 2a. Among phage types of thestrains originating from the G.D.R., 25, 27 an d 44 were rare in Hungary (0.2, 0.3, 0.4%, respectively), while the occurrence of 33, 37, 42 and 43 was frequent in our country too (0.6, 1.1, 9.4, 0.8, respectively). Two strains were of serotype 5 and phage type 27 — this type was found in serotype 3a in Hungary. Serotype 5 was rare in our material, as only two strains isolated

in Hungary were found during four years. Their phage types were 16 and 84, and this might explain their low frequency.

Strains from Bulgaria were of serotype 2a, phage type 69 and 73. The frequency of these types was 1.7% and 0.9%, respectively, in Hungary.

Analyzing the phage and serotypes of strains responsible for outbreaks, we found serotype 3a phage type 5 and 42 responsible for 1 and 2 outbreaks, respectively. In 1969, the number of phage type 5 increased, 7 outbreaks were caused by this type. In 1970 it was overcome by phage type 19; this was isolated from one local and 3 institutional outbreaks.

Analyzing the occurrence of serotype 2a, the most frequent phage types were 22 and 16. Type 22 caused two outbreaks in 1968, one in 1969 and one in 1970. Phage type 16 was the epidemic strain once in 1968 and in 1970, and twice in 1971. Phage types 71 and 76 were spread in 1971, 3 (2 and 1) outbreaks were caused by them.

Strains of serotype 4a: phage type 81 caused four outbreaks in 1969, and two in 1971. In 1970, strains of phage type 89; in 1971, phage types 78 and 83 caused outbreaks.

Serotype 6: two outbreaks each were caused by strains of phage type 85 in 1968, 1969 and in 1971.

Among 1b serotypes, in 1968, strains of phage type 59, in 1970, type 73, were isolated from outbreaks. Outbreaks were caused by var. X in two cases — in 1969 by type 32, in 1970 by phage type 28.

The strains originating from 7 local and 47 institutional outbreaks in 31 cases were homogeneous in phage type. In other cases one or two other serotypes or phage types occurred besides the dominant epidemic strains.

There are two explanations for this observation. Strains o riginating from control examinations were examined too, but these strains were not connected with epidemics, as shown by the presence of different serotypes apart from different phage types.

Another possibility is the alteration of phage types. These changes in phage sensitivity might be a result of some alteration in the lysogenic state of the strain. Another possible effect is that the strain may have been infected with plasmids, for example with R or col-factors.

During the outbreaks, the phage type proved stable, although strains of different sensitivity to phages and antibiotics were isolated from one and the same person in repeated control examinations.

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INHIBITION OF VIRUS MULTIPLICATION BY PARTIALLY HYDROLYSED RNA: A POSSIBLE MEMBRANE-EFFECT

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Summary. Effects of 1–10 mg/ml partial RNA hydrolysate concentrations were examined in different tissue culture–virus systems. Virus adsorption, multiplication of cells and enveloped viruses, and cellular uptake of neutral red were found to be inhibited. Possible membrane-targets seemed to be involved in the inhibition of multiplication of cells and enveloped viruses by RNA hydrolysate at high concentrations.

Different types and derivatives of nucleic acids exert heterogeneous effects on mammalian cell functions and viral multiplication. Rate of cell division, immune responses [1] and rate of virus multiplication are influenced either directly [2, 3] or indirectly, due to induction or inhibition of interferon synthesis [4, 5]. Even single-stranded preparations of RNA may interfere with virus multiplication under certain conditions [6] and non-inducer RNA types can be rendered active by partial enzymatic hydrolysis [7]. Wide ranges of doses and polycation additives have been used for cell treatment, although these are unable to protect RNA from partial enzymatic degradation, but alter the physicochemical state of molecules [8] and may play an essential role in the mechanism of interferon induction [9].

To avoid the disturbing presence of additives and diminish the rarely discussed role of RNA degrading enzymes present in tissue cultures, experiments have been performed using high concentrations of partially hydrolyzed yeast RNA.

Materials and methods

Reagents. A stock solution of 100 mg/ml was prepared from commercial yeast RNA (free acid, EGA Chemie K. G., G.F.R.) converted to potassium salt and hydrolyzed either by 0.3 M potassium hydroxide (20 hr at 37°C) or by 4 mg crystalline pancreatic RNase per g (Reanal, Budapest, 1900 EU/mg). The pH was adjusted to 7.2–7.4 by potassium hydroxide and/or perchloric acid. All solutions were filtered through Millipore GSWP filters before storage at —40°C. The percentage of mono and dinucleotides was determined by DEAE-cellulose chromatography [10] and calculations were done on the basis of the extinction values measured at 260 and 280 nm in a Beckman DU spectrophotometer. The alkaline hydrolysate contained less than 2.7% phosphateless breakdown products, about 7.0% mononucleotides and about 14.0% dinucleotides eluting in the first 15 peaks.

RNA derivatives were incorporated either into Parker's 199 medium or into agar overlay as described by DÖMÖK and SIMON [11], but neutral red was omitted. Instead of vital dye, Giemsa staining was used after formalin fixation.

Neutral red, NBC Research Chemicals, (20 μg/ml); dimethyl sulphoxide, DMSO, Fluka A.G., G.F.R.; 5'AMP-Na_2, Zellstoffabrik Waldhof; 2'3'AMP, Sigma Chem. Co. (converted to potassium salt); ATP-$Na_2 \cdot 4H_2O$, Reanal, Hungary, were used throughout the experiments.

Cell cultures. HeLa, primary human embryonic kidney (HEK), primary rhesus monkey kidney (PMK) and permanent rhesus monkey kidney cells (III/1 of RUZICSKA [12]) were grown in tube cultures, ampoules of about 2 cm^2 flat surface and in plastic Petri dishes (TC-4 and TC-6, LINBRO Chemical Company, Inc., New Haven, Conn.).

Virus strains. Herpes simplex virus (giant-cell type) isolated by, and received from, Dr. J. LESSO, Bratislava; vaccinia virus Budapest strain (3–6 PMK passage); influenza virus A/H3N2/69 and echovirus type 7 of haemagglutinating character (H^+), both isolated on PMK cells by Dr. M. SIMON, Budapest; poliovirus type 1, Mahoney strain; poliovirus type 3 strains Saukett, "30", Usol-D-bac, Leon $12a_1b$ and adenovirus type 1.

Results

In contrast to the finding of FUKADA *et al.* [7] the tissue cultures survived 2–9 days incubation under 4–10 mg/ml RNA hydrolysate at 37°C without much damage as observed under the light microscope. As with the inhibition caused by nucleosides [2], reversible inhibition was observed in tube cultures of both HeLa and PMK cells (Table I). The increase in number of

Table I

Effect of partial alkaline hydrolysate of RNA on cell counts of (a) HeLa and (b) PMK monolayers

Cell type and incubation medium	Days after seeding cells	RNA present in incubation medium	Cell count/tube × 10^4	
			Treated	Control
a	0	—	8	8
HeLa, Parker's 199 with 10%	1	—	.	.
bovine serum	2	4 mg/ml (24 hr)	14	14
	3	—	17	31
	4	—	41	45
	5	—	51	60
b	0—7	—	36	36
PMK cells, Parker's 199	8	9 mg/ml	.	.
	9	9 mg/ml	44	48
	10	9 mg/ml	.	.
	11	9 mg/ml	48	67
	12	9 mg/ml	.	.
	13	9 mg/ml	.	.
	14	9 mg/ml	54	73

Each figure represents cell counts for 5–10 tubes detached by 0.1% versene solution and stained with 0.1% methyl violet dissolved in citric acid

. = Not tested

morphologically intact cells was restricted during the presence of alkaline-RNA-hydrolysate. In separate experiments, not shown in Table I, permanent III/1 cells started dividing even after 9 days incubation under 10 mg/ml RNA if the medium had been changed for Parker's 199 supplemented with 10% bovine serum.

Failure in uptake of neutral red, reported by FUKADA *et al.* [7], is not a consequence of the loss of viability; it is caused by an inhibition of dye uptake with both polymer and hydrolysed RNA. The inhibition was shown to be proportional to the RNA concentration in the medium. A reversed phenomenon was also observed. Cell monolayers were incubated in the presence of neutral red (20 μg/ml) and they took up 3.5–5 μg/cm^2. They were then washed three times with saline and incubated with a saline-containing dilution of RNA or RNA hydrolysate. The dye was released at 37°C in the dark in proportion to the RNA concentration as estimated 90 min later at 530 nm (Fig. 1). A similar release of dye was observed after treatment with dimethyl sulphoxide. All cultures were shown to support multiplication of vaccinia virus after the described release of neutral red.

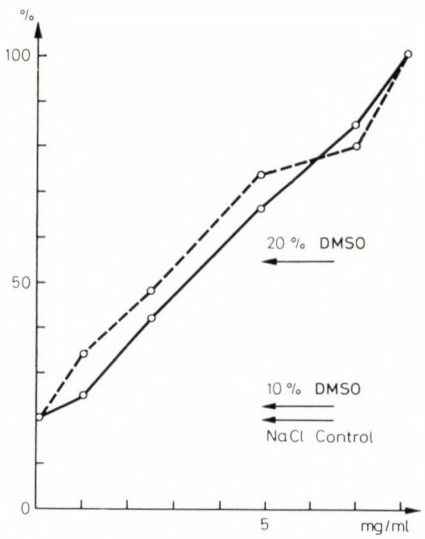

Fig. 1. Release of incorporated neutral red on treatment of cells with polymer RNA, pancreatic RNase digest of RNA and DMSO. Ordinate: released neutral red, %; abscissa: concentration of RNA, mg/ml. Arrows show the percentage of dye released on treatment of DMSO concentrations indicated. Enzymatic digest o———o; polymer o---------o

Effects of different RNA and nucleotide solutions were tested on the multiplication of the listed viruses. Partial RNA hydrolysate incorporated into the incubation media was found to reduce dilution end point and/or

plaque numbers only in the case of viruses which possess a membrane on their surface (Table II). Subsequent log virus yield from cultures with identical

Table II

Log decrease in plaque number or dilution end-point and of virus yield, compared to control cultures, due to the effect of RNA partial hydrolysate on virus strains tested

Virus strain	Tissue culture	Decrease in plaque number or dilution endpoint under RNA	Decrease in virus yield
Herpes simplex	HeLa	2.2–3.0 (2–5)*	1.7–3.7 (3–6)
Vaccinia	PMK or III/1	2.2–4.5 (5–10)	0.7–5.0 (5–10)
Influenza A2	PMK	.	3.7 (10)
Echo type $7H^+$	PMK	.	0.0–1.0 (7–10)
Mahoney	PMK	0.0 (3–10)	.
Leon $12a_1b$	PMK or III/1	0.0 (8)	0.5 (10)
Usol-D-bac	PMK or III/1	0.0 (8)	0.5 (10)
Saukett	III/1	0.0 (10)	.
Adenotype 1	HEK	.	0.0 (10)

See text for details and abbreviations.
* In brackets: concentration range of RNA hydrolysate, mg/ml
. = Not tested

inoculum (10–10 000 $TCID_{50}$) was assayed in most of the experiments. Decrease of virus yields in the presence of alkaline RNA hydrolysate, compared to the control values, is summarized in Table II. Significant inhibition could be observed only with herpes, vaccinia and influenza A2 viruses. Picornaviruses and the DNA virus without envelope used in this test, multiplied in the presence of RNA hydrolysate as well as in Parker's 199 medium.

Comparison of haemagglutinin formation titrated by TAKÁTSY's microtechnique [14] revealed similar differences. In the case of echovirus type 7 H^+ (human O RBC suspension) there was only a twofold reduction of haemagglutinin formation by RNA hydrolysate as compared to the eightfold decrease observed with influenza virus (guinea pig RBC suspension, 8 mg RNA hydrolysate per ml).

Direct inactivation of the viruses by 10 mg RNA hydrolysate per ml was excluded, since there were no significant differences in plaque counts of vaccinia virus after 48-hr incubation at 37°C and three cycles freezing-thawing of the same dilutions in the presence and absence of that concentration.

The same difference (*i.e.*, inhibitory effect only on multiplication of enveloped viruses) was observed in experiments with pancreatic RNase digest, 5'AMP · Na_2, 2'3'AMP · K_2 and ATP · Na_2 · $4H_2O$. Control experiments with 0.15 M Earle's salt solution or 0.15 M potassium chloride added to Parker's 199 excluded the role of a hyperosmotic environment in the inhibition of vaccinia virus multiplication, since the molarity of 10 mg/ml RNA solution in distilled water was only 0.026 M as calculated for a mixture of mononucleotides.

The RNA derivatives reduced adsorption of both vaccinia and enteroviruses at both 37°C and 4°C. Results of an experiment on III/1 cell monolayers grown in Linbro TC-6 Petri dishes are listed in Table III. The decrease

Table III

Reduction of plaque count by RNA derivatives present only during the 3— hr adsorption period in an experiment with vaccinia and Usol-D-bac viruses

RNA derivative and concentration	Vaccinia*	Usol-D-bac*
2'3'AMP-K_2 0.01 M	52	72
5'AMP-Na_2 0.009 M	53	28
Partial alkaline hydrolysate 0.01 M**	33	81
intact RNA 0.01 M**	38	69
Control, Parker's 199	100	100

* Per cent of control plaque counts
** Molarity calculated for the mixture of mononucleotides

in vaccinia plaque counts was more pronounced probably because of the long adsorption period (60 min at 24°C and 120 min at 37°C with 2 ml inocula). As with anionic and cationic polymers [12, 15], 40–60% inhibition was regularly observed. This effect, however, was not concurrent with the extreme reduction seen in Table II.

Direct inhibition of influenza haemagglutination could also be achieved, but 33 mg/ml hydrolysate concentration gave only a fourfold reduction in titre with chick RBC system.

In tests with agar overlay incorporating different concentrations of RNA solutions (Fig. 2), the same 50% plaque reducing concentrations were calculated for 5'AMP, and for partial alkaline hydrolysate which contained only about 21% mononucleotides. This value was found in all experiments with III/1 monolayers and vaccinia virus to be between 1 and 2 mg/ml concentrations (0.003–0.006 M). As expected, values for poliovirus strains and with polymer RNA solution [6, 7] in both vaccinia and poliovirus systems, were found to be higher than 8 mg/ml (0.021 M).

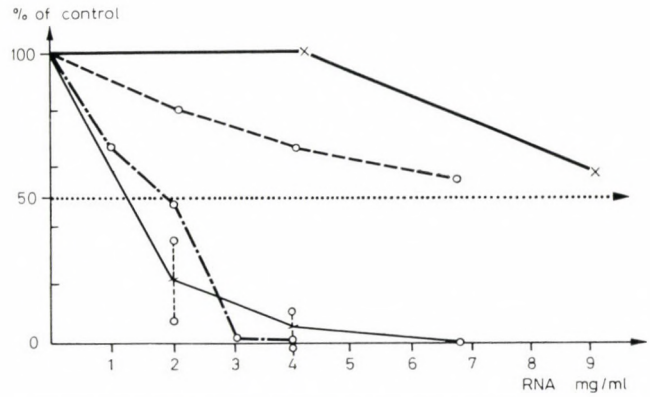

Fig. 2. Plaque count reduction of vaccinia virus and poliovirus strain "30" by 5'AMP, partial alkaline hydrolysate of RNA and original non-hydrolysed RNA incorporated into the agar overlay. Ordinate: plaque counts in percentage of control; abscissa: concentration of compounds in mg/ml. Poliovirus + partial hydrolysate: ×——×; vaccinia virus + partial hydrolysate: o——o; vaccinia virus + polymer solution: o------o; vaccinia virus + 5'AMP-Na_2 solution: o·····o

Discussion

The reversible inhibition of cell multiplication seems to be similar to that observed with nucleosides [2], but cannot be produced by contaminating phosphateless breakdown products. The amount of uridine and uracil, described as most active [2], is calculated to be less than 0.2 mM in a 10 mg/ml solution of alkaline hydrolysate.

The conclusion of GIFFORD [3], that both RNA and DNA viruses are inhibited by nucleotide mixtures, proved to be valid only for enveloped viruses. In contrast, adsorption of both virus groups is influenced by the same rate similar to the effect of other ionic polymers, described in several papers [12, 15, 19].

These activities are different from the actinomycin-like effect of adenine [5], since adenovirus type 1 multiplies well in the presence of RNA oligomers, and Chikungunya virus, a virus rather resistant to the drug, may be inhibited [3].

The influence of RNA oligomer mixtures on virus adsorption, on multiplication of enveloped viruses and on host cell metabolism is considered to be unrelated phenomena elicited by the same treatment. The role of cell membrane in regulation of macromolecular metabolism has been suggested [4, 17, 18, 19] and the results seem to support the view that RNA-membrane interaction is the main and/or single cause of the three above phenomena.

The cellular uptake of macromolecular RNA is extremely low at pH 7.2 without additives [16, 17]. Owing to the slow penetration of oligonucleotides one would expect significant differences between 50% plaque number reducing concentrations of full and partial hydrolysates. This was not the case.

The influence of oligonucleotides on regulatory membrane functions is also suggested by the fact that neutral red release was found to be identical following treatment with polymer and digested RNA but the inhibitory effect on vaccinia virus multiplication proved to be different.

The role of an oligomer influx upon permeability changes seems to be improbable, since heteropolyacid polymers inhibit the multiplication of vesicular stomatitis virus [19] and cell multiplication is inhibited only by DEAE-dextran treatment [18]. These two observations support the possibility that aspecific alterations of the functional state of the cellular membrane may be the cause of specific changes in cellular metabolism.

The supposition of specific membrane targets, which would possess a regulatory function on intracellular processes, had been described in connection with cyclic AMP metabolism [20]. An influence on the function of these and of possible other regulatory sites seems to be the most feasible explanation for the diverse effects observed with different RNA solutions. For this suggestion, a theoretical and experimental basis has recently been provided by MÉCS, who found a mutuality in the regulation of interferon production by macromolecular inducers and substances activating the cyclic AMP system [21].

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EFFECT OF CYCLIC AMP AND POLYADENYLIC ACID TREATMENT ON THE DELAYED HYPERSENSITIVITY OF GUINEA PIGS

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(Received December 13, 1972)

Summary. Prolonged cAMP treatment was found to stimulate delayed hypersensitivity in the guinea pig. Continuous treatment with single-stranded PolyA stimulated the delayed type of allergic dermatitis to a greater extent than did cAMP treatment representing an equal amount of adenylic acid. The possibility of desensitization in animals treated with cAMP or PolyA is similar. Both cAMP and PolyA were found to enhance the macrophage disappearance reaction.

There are several data to prove that the humoral and cellular immune reaction can be stimulated by synthetic polynucleotides (PolyA : U, PolyI : C) activating adenyl-cyclase. These experiments had in common that all authors applied double-stranded polynucleotides in systems *in vivo* to defend the synthetic preparations from ribonuclease, and they all examined the effect of a single treatment [1—5].

In delayed hypersensitivity, a significant role is attributed to nucleic acids and their derivatives [6, 7] on account of the characteristics of the hypothetic mediator, the permeability factor described by WILLOUGHBY *et al.* [6].

The present experiments were aimed at studying the following questions.

(a) How does a continuous cyclic AMP (cAMP) treatment affect the delayed hypersensitivity?

(b) Is the continuous application of the single-stranded synthetic polynucleotide polyadenylic acid (PolyA) effective *in vivo?*

(c) What are the possibilities of desensitization in animals treated with cAMP or PolyA?

(d) What effect has cAMP or PolyA treatment on the macrophage disappearance reaction, the reaction characteristic of delayed hypersensitivity?

Materials and methods

Animals. White female guinea pigs, weighing 300—350 g.

Sensitization [8]. Each hind paw of the animals was injected with 3 μg of twice recrystallized ovalbumin in 0.1 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.).

Skin testing. On the 8th day after sensitization, the guinea pigs were given intradermally 6 μg twice recrystallized ovalbumin dissolved in 0.1 ml of physiological NaCl; 24 hr later the skin was examined for the inflammatory reaction and its area. The Figures illustrate the mean

size in mm^2 of the reactive area in each animal group. The ovalbumin was injected at two sites into the clean-shaven back skin of the animals.

Desensitization [6, 9, 10]. On the 7th day after sensitization, *i.e.* 24 hr before the testing injection, the animals were given 4 mg of twice recrystallized ovalbumin intraperitoneally. To avoid anaphylactic shock, both the experimental and control animals were given 2 mg of chloropyramine

3'-5'cAMP was prepared from 5'AMP (Reanal, Budapest) at the Institute of Biochemistry, University Medical School, Debrecen. The product was chromatographically homogeneous and resistant to *Escherichia coli* alkaline phosphatase. The phosphorus content of the preparation was 8.3%. The treated animals were given intraperitoneally 1 mg of cAMP dissolved in 0.5 ml saline daily, from the day of sensitization until the termination of the readings. The controls were treated with 0.5 ml of saline daily.

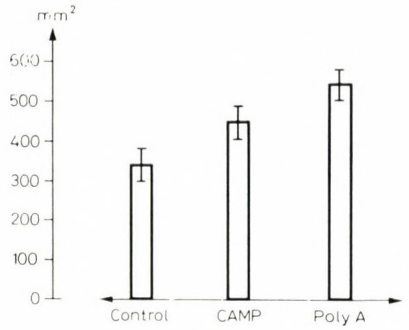

Fig. 1. Effect of continuous cAMP and PolyA treatment on size of inflammatory area in delayed hypersensitivity of guinea pigs. Mean of 5 experiments

Polyadenylic acid (Reanal, Budapest): mol wt 14 000; phosphorus content, 9.2%. The treated animals were given 0.9 mg of PolyA (representing an amount of adenylic acid equal to the amount of cAMP given) dissolved in 0.5 ml of saline, from the day of sensitization until the termination of the readings. The controls were given 0.5 ml of saline intraperitoneally.

Provocation of peritoneal exudation. Five days before the induction of the reaction, the guinea pigs were given 10 ml of liquid paraffin intraperitoneally.

Preparation of macrophages. 5 days after giving liquid paraffin, the animals were bled-to death by cutting the carotid, and 20 ml of Hanks solution was injected into their abdomen. The exudate was aspired, then centrifuged twice at 200 *g* and 4°C in Hanks solution. After fixation of the deposit with methanol and staining with Giemsa, quantitative and qualitative cell counts were made.

Macrophage disappearance reaction [9]. On the day of the experiment, the animals were injected intraperitoneally with 10 μg of twice recrystallized ovalbumin in 1 ml of Hanks solution. The controls were given Hanks solution only. Four hr after the injection the peritoneal exudate was examined for the number of cells and for the ratio of the different cells: macrophage/polymorphonuclears and lymphocytes. Five guinea pigs were used in each experiment.

Results

The size of the reactive area of delayed hypersensitivity was increased by both cAMP and PolyA treatment, somewhat more by PolyA (Fig. 1). Desensitization significantly decreased the size of the reactive area both in the controls and in the animals treated with PolyA. Comparing the inflammatory area of the original (not desensitized) animals with that of the desensitized animals, the degree of desensitization was proportional in all the three groups.

The decrease was 3—3.5-fold. Therefore the inflammatory area was the largest in animals treated with PolyA, smaller in animals who were given cAMP and smallest in the controls (Fig. 2).

Examining the macrophage disappearance reaction 4 hr after the injection of 10 μg of ovalbumin antigen the number of cells prepared from the exudate was $7—9\times10^6$/ml; 72—74% of them were macrophages, the rest polymorphonuclear leucocytes and a few lymphocytes. The relatively high leucocyte count was believed to be due to the repeated intraperitoneal injections.

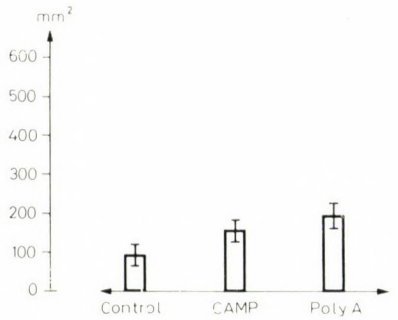

Fig. 2. Effect of continuous cAMP and PolyA treatment on size of inflammatory area in delayed hypersensitivity of desensitized guinea pigs. Mean of 5 experiments

The macrophage count was decreased to a greater extent in the animals treated with cAMP or PolyA than in the untreated controls. No significant difference was found between the effect of cAMP and PolyA treatment on the macrophage disappearance reaction (Table I).

Table I

Effect of cAMP and PolyA treatment on the macrophage disappearance reaction

Group and No. of animals	Absolute macrophage count	
	Before antigen	4 hr after antigen
Control (5)	$5.7 \pm 0.7\times10^6$	$3.4 \pm 0.4\times10^6$
cAMP treated (5)	$5.7 \pm 0.7\times10^6$	$2.3 \pm 0.3\times10^6$
PolyA treated (5)	$5.7 \pm 0.7\times10^6$	$2.2 \pm 0.25\times10^6$

Discussion

According to data in the literature [11—13] the mode of action of cAMP in the different immune processes has two pathways. First, the activity of thymocytes and lymphocytes is increased and, second, certain processes of protein-synthesis are increased by the phosphorylation of histones.

In our experiments the delayed hypersensitivity was increased by continuous treatment with cAMP or PolyA.

According to the above, the phenomenon was due to the activation of the thymus dependent lymphocytes, which play a major role in this type of inflammation [5].

The difference observed in the enhancement of the reaction by cAMP and PolyA in amounts representing an identical quantity of adenylic acid, was likely due to the difference in the decomposition rate of the two substances. cAMP is split by phosphodiesterase, PolyA by ribonuclease, and cAMP seems to be decomposed at a higher rate than is PolyA. The oligonucleotide products of the latter also have an adenylic-cyclase activity increasing effect [14, 15].

The possibility of desensitization was similar in both the treated and the control groups and the reactive area decreased to the same degree in all three groups.

By means of the macrophage disappearance reaction it was possible to prove on a cellular level the increased sensitivity to the specific antigen of the animals treated with cAMP or PolyA.

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REPRESSION OF ALKALINE PHOSPHATASE SYNTHESIS BY ADENINE, ADENOSINE AND ADENINE NUCLEOTIDES IN AN ADENINE AUXOTROPH OF BACILLUS ANTHRACIS

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(Received December 27, 1972)

Summary. Further evidence of a relationship between the genes regulating alkaline phosphatase and the structural genes of purine synthesis has been obtained in *Bacillus anthracis*. Adenine, adenosine, and adenine nucleotides repressed the production of alkaline phosphatase in an adenylosuccinate lyase deficient mutant of *B. anthracis*. During the growth phase, the ATP level was lower in the mutant than in the prototroph. The adenine nucleotide pool was supposed to act as co-repressor in the regulation of enzyme production. Exogenous cyclic AMP also exhibited a repressor effect though it was not detectable in any of the *B. anthracis* strains studied.

The rate of alkaline phosphatase synthesis in *Bacillus subtilis* and *Bacillus cereus* is a function of the anorganic phosphate concentration in the medium. The repressor effect of orthophosphate on the synthesis of the alkaline phosphatase asserts itself more explicitly in *Bacillus anthracis* than in *B. cereus* strains producing higher amounts of the enzyme [1]. Phosphatase production proved useful in the differential diagnosis of the two bacterial species closely related phylogenetically [2]. In our laboratory, a number of auxotrophic mutants have been isolated from the acapsulogenic strain Vollum of *B. anthracis* [3]. Studies of alkaline phosphatase regulation in the auxotrophs showed that on the basis of their orthophosphate repressibility, the mutants could be divided into three groups [4]. Those in the first group showed a behaviour identical to that of the prototroph. Some pyrimidine and vitamin dependent mutants and purine dependent mutants in which the block occurred before the closing of the ring, belonged to this group. In these strains, a gradual increase in phosphate concentration was paralleled by a decrease in enzyme synthesis. In the second group, a two-phase repression was observed in strains deficient in the synthesis of adenylosuccinate synthetase and adenylosuccinate lyase. Alkaline phosphatase synthesis in some guanine dependent mutants was not repressed even at low phosphate concentrations. The latter belonged to the third group.

The capsulated variants of the adenine dependent strains failed to prove pathogenic in animal experiments [5].

Electron microscopic observations showed an accumulation of alkaline phosphatase in the space between the cytoplasmic membrane and cell wall in

the adenine dependent strain. Accumulation of the enzyme was accompanied by a damage or a loosening of the structure of the cell wall [6].

These findings have prompted us to study whether the loss of virulence in the adenine dependent mutant was associated with an enhanced production of alkaline phosphatase. The relationship between adenylosuccinate deficiency and the derepression of alkaline phosphatase was also investigated.

Materials and methods

Bacterial strains. The non-capsulogenic prototrophic strain Vollum (VR) of *B. anthracis* and its adenine auxotrophic derivate, ade II-3, deficient in adenylosuccinate lyase [5] denoted as $23C^-$ ade^-, were used. Studies were performed also on the B strain of *Escherichia coli.*

Medium. Lactalbumin hydrolysate was supplemented with proper amounts of glucose, vitamin B_1, and various salts [4]. The medium was prepared according to the following modification: after the dephosphorylation of lactalbumin hydrolysate by barium hydroxide [6], the basic medium was supplemented and then a proper amount of phosphate was added from a 100 mM solution of K_2HPO_4.

Cultivation. After the addition to the lactalbumin of orthophosphate and adenine the medium was divided in 10 ml aliquots into 100 ml Erlenmeyer flasks and then each of the flasks was inoculated with 10^5 spores. The flasks while aired in a reciprocal shaker, were incubated in a water bath at 37°C for 16 hr.

Measurement of optical density. The optical density of the cultures was measured in a Magnephot II apparatus (Orion, Budapest).

Measurement of alkaline phosphatase activity. The bacterial cultures were centrifuged in cold at 3500 rpm for 15 min. The pellet was washed once and then resuspended to OD 0.6 in physiological saline. To 2 ml bacterial suspension, 2 ml reagent containing 0.5% paranitrophenyl phosphate in 0.2 M Tris-HCl buffer pH 8.5, was added. The samples were incubated in a water bath at 37°C for 20 min. After incubation, the enzyme reaction was stopped by 1.0 ml 1.0 N NaOH. The suspensions were centrifuged and then the extinction values of the supernatants were measured by a Spectronom 201 spectrophotometer at 410 nm. Unit of enzyme activity was expressed in terms of 1.0 mμmol paranitrophenol released by 1.0 ml bacterial suspension of OD 0.6 during 20 min.

Repression of alkaline phosphatase synthesis. Series of lactalbumin medium were prepared by the addition of varying amounts of 0.1—1.6 mM phosphate. The amount of adenine varied between 10 and 100 μg/ml. The medium also contained nucleosides and nucleotides in amounts varying between 0.25—1.0 mM.

The inhibitory effect of some enzymes on the alkaline phosphatase synthesis was studied also in a medium containing 1.0 mM orthophosphate and 10 μg/ml adenine. Cyclic AMPphosphodiesterase inhibition [7] was studied with caffeine and theophylline while adenylcyclase inhibition [8] with chlorpromazine (Hibernal, EGYT Pharmacochemical Works, Budapest).

Furthermore, 2'4-dinitrophenol was also used as an uncoupling agent. The alkaline phosphatase activity of *B. anthracis* cultures prepared conventionally in media containing enzyme inhibitors, was determined.

Detection of 3,5-cyclic adenosine monophosphate. The prototrophic and adenine dependent strains of *B. anthracis* were cultivated in the presence of 1.0 mM K_2HPO_4 and 10 μg/ml adenine in lactalbumin medium. For the cultivation of the B strain of *E. coli*, the medium described by MAKMAN and SUTHERLAND [9] was used. From the cultures 4 g of the sediment was resuspended in 20 ml cold 0.05 N HCl and then incubated in a water bath at 90°C for 5 min. After centrifugation, the supernatants were decanted and neutralized to pH 7.4 with NaOH. The precipitate formed during neutralization was centrifuged again. The pure supernatant was concentrated to 5.0 ml by freeze-drying. After thawing, 0.3 ml of 0.3 M $ZnSO_4$ and 0.3 ml of 0.3 N $Ba(OH)_2$ were added and the precipitate formed was removed by centrifugation. This procedure was repeated to remove non-cyclic nucleotides [10]. The samples were desalinized on Dowex 50 H^+ (100—200 mesh) columns of 0.6×3.0 cm size [11]. Chromatography was performed on Silicagel GF_{254} plates [11]. The plates were dried by cold air and detected under an UV lamp. The cyclic AMP spot ($R_F = 0.39—0.41$) was scraped off, resuspended in 3.0 ml deionized water and stored at 50°C. Cyclic AMP was identified according to its spectra taken in the range of 220—280 nm at various pH values [12].

Estimation of adenosine triphosphate in growing cultures of B. anthracis. Spores of the prototrophic and adenine dependent strains were inoculated into medium containing 1.0 mM K_2HPO_4 and 50 μg/ml adenine and cultivated in the conventional way. The optical density of the cultures was determined and 2 ml samples were taken every hour. After the addition of 2.0 ml cold 0.5 N perchloric acid, the samples were incubated under constant stirring in an ice bath for 30 min. After centrifugation, the supernatants were neutralized by 10.0 N K_2CO_3 [13]. The precipitate formed was centrifuged and the ATP content of the supernatants was determined by luciferase [14].

A 5 mg/ml solution of luciferase (Firefly Lantern extract, Serva, Heidelberg) was prepared in deionized water. For the determination of ATP, 0.4 ml deionized water, 0.1 ml enzyme solution, and then 0.1 ml bacterial extract were measured into an absorption cell. Measurements were started 15 s after the addition of the bacterial extract containing ATP and continued for 1 min. Counts per 1 minute were determined by a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 2001. As a standard, 0.1 ml each of different dilutions of a 5×10^4 mM/ml ATP stock solution added to the enzyme, was used.

Results

The prototrophic strain and the adenine dependent mutant of *B. anthracis* showed suboptimal growth and a high alkaline phosphatase activity at concentrations of 10 μg/ml of adenine and 0.1 mM of phosphate. The rate of enzyme synthesis could be repressed to a constant low level by increasing the concentration of anorganic phosphate. The amount of anorganic phosphate needed for repression was 0.8—1.0 mM for the prototrophic strain while under the same conditions enzyme synthesis was still derepressed in the mutant requiring 1.6 mM phosphate for repression. When the amount of adenine was increased to 50 and 100 μg/ml in the medium repression occurred in the auxotrophic strain at 0.5—0.8 mM phosphate concentration. Increasing the amount of adenine failed to influence enzyme production by the prototrophic strain (Fig. 1).

Further experiments were performed in a medium containing 1.0 mM anorganic phosphate. Under such conditions, enzyme synthesis was repressed in the prototrophic strain while it was still derepressed in the adenine dependent mutant. The medium was supplemented with 10, 25, 50, and 100 μg/ml adenine. When bacteria were cultivated in media thus completed, an enhancement of enzyme repression was observed parallel to the increase in the amount of adenine. In the presence of 50 μg/ml adenine, enzyme production of the mutant was essentially the same as that of the prototrophic strain (Fig. 2).

Since alkaline phosphatase synthesis in the adenine dependent mutant could be repressed not only with anorganic orthophosphate but also with adenine, the effects of some other purine compounds were also tested. For these studies, media containing 1.0 mM phosphate and 10 μg/ml adenine were supplemented separately with 10—100 μg/ml of adenosine and inosine. Of the compounds studied, only adenosine exerted a moderate repression on enzyme synthesis by the mutant (Fig. 3).

Inosine proved to be ineffective at the concentrations used. It seemed that adenine at a low concentration was not able to support an appropriate synthesis of some adenine nucleotide of regulator effect. Consequently, in the

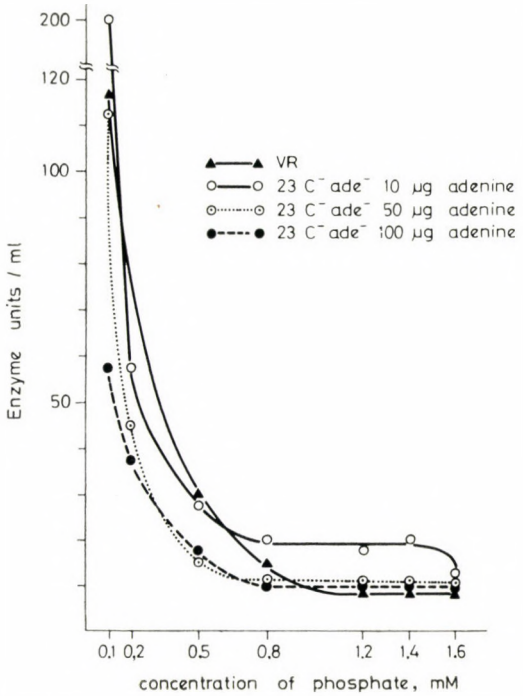

Fig. 1. Effect of orthophosphate and adenine concentrations of the medium on alkaline phosphatase activity of the prototrophic (VR) and adenine auxotrophic (23C⁻ ade⁻) mutants of *B. anthracis*

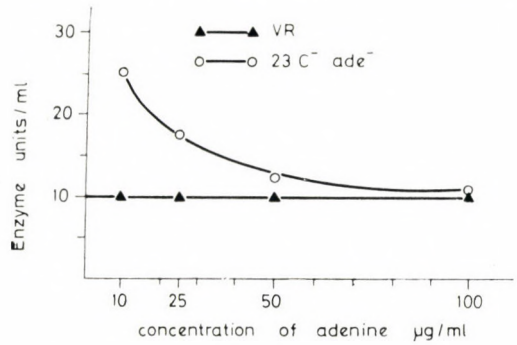

Fig. 2. Repression by adenine of alkaline phosphatase synthesis in the prototrophic (VR) and adenine auxotrophic (23C⁻ ade⁻) strains of *B. anthracis*

next experiments, media containing 1.0 mM phosphate and 10 μg/ml adenine were supplemented with 0.25—1 mM cyclic-AMP, AMP, ADP, ATP, and IMP. The prototrophic and adenine auxotrophic strains were cultivated in the above media and then the alkaline phosphatase activity of the cultures was determined. The cyclic-AMP, AMP, and ADP concentration dependent repressors

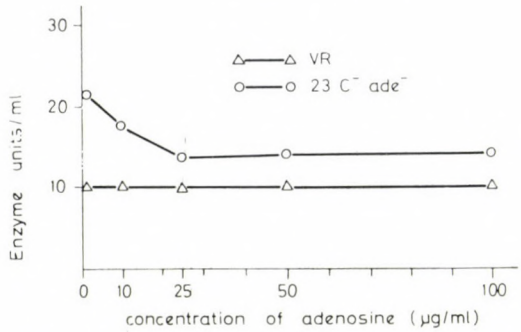

Fig. 3. Repression by adenosine of alkaline phosphatase synthesis in the prototrophic (VR) and adenine auxotrophic (23C$^-$ ade$^-$) strains of *B. anthracis*

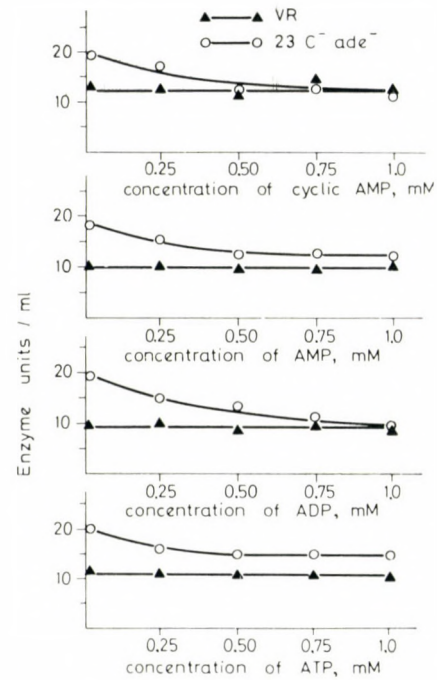

Fig. 4. Repression of alkaline phosphatase synthesis by cyclic-AMP, AMP, ADP, and ATP in the prototrophic (VR) and the adenine auxotrophic (23C$^-$ ade$^-$) strains of *B. anthracis*

exerted comparable effects on enzyme synthesis. ATP at 0.25 mM concentration similarly as the earlier studied nucleotides, led to a partial decrease in enzyme production while a further increase in the amount of ATP had no influence on enzyme repression.

Nucleotide concentrations of 0.25—0.5 mM induced a repression of enzyme synthesis in the mutant (Fig. 4).

The type of the repression curve of inosine monophosphate was different from that of adenine nucleotides. Inosine monophosphate at concentrations of 0.75—1.0 mM caused a small decrease in the mutant's enzyme production (Fig. 5).

Enzyme synthesis by the prototrophic strain was not influenced by the nucleotides studied. The repressor effect of cyclic-AMP prompted us to study the effect of caffeine and theophylline inhibiting cyclic-AMP-phospho-

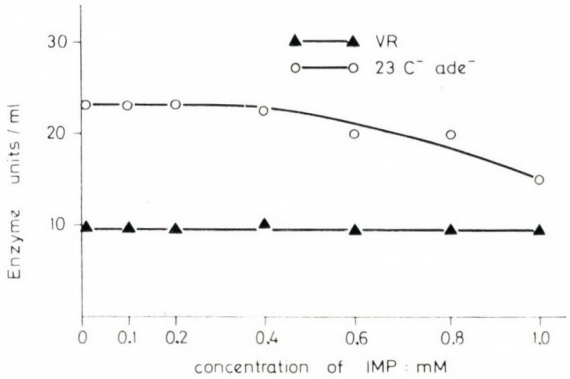

Fig. 5. Repression by IMP of alkaline phosphatase synthesis in the prototrophic (VR) and adenine auxotrophic (23C⁻ ade⁻) strains of *B. anthracis*

diesterase, and of chlorpromazine inhibiting adenylcyclase, on the enzyme production of the two strains. Caffeine and theophylline at concentrations of 1.0—5.0 mM enhanced the derepression of alkaline phosphatase in both strains. Chlorpromazine at a concentration of 1.0 mM completely inhibited the propagation of bacteria. Consequently, concentrations of 0.008—0.040 mM were used which had practically no effect on the growth of the strains studied. Chlorpromazine caused a moderate repression in the adenine auxotroph while it had no effect on enzyme synthesis by the prototrophic strain (Fig. 6).

In order to study the specificity of the antibacterial effect of chlorpromazine, agar diffusion experiments were performed. It was found that chlorpromazine exerted a bacteriostatic effect when present in amounts of 12—25 μg/ml.

The differences in the effect of cyclic-AMP and various enzyme inhibitors made its specificity questionable. Consequently, the direct detection of cyclic-AMP was attempted from the prototrophic and adenine dependent mutants of *B. anthracis* and *E. coli* B used as control. Identification of cyclic-AMP from purified bacterial extracts was made by thin-layer chromatography. After elution of the spot, further identification was made by spectrophotometry. While no cyclic-AMP could be detected in the prototrophic and the adenine dependent mutants of *B. anthracis*, 85 μg cyclic AMP-could be isolated

from the cultures of *E. coli* B. The spectrum of the isolated cyclic-AMP was taken in the range of 220—280 nm at pH 1.0, 7.0, and 12.0.

These results spoke against the role of cyclic-AMP in the alkaline phosphatase synthesis by *B. anthracis*. It has therefore been concluded that other adenine nucleotides must have a role in physiological regulation. The effect of the uncoupling agent 2'4-dinitrophenol on enzyme synthesis by the prototro-

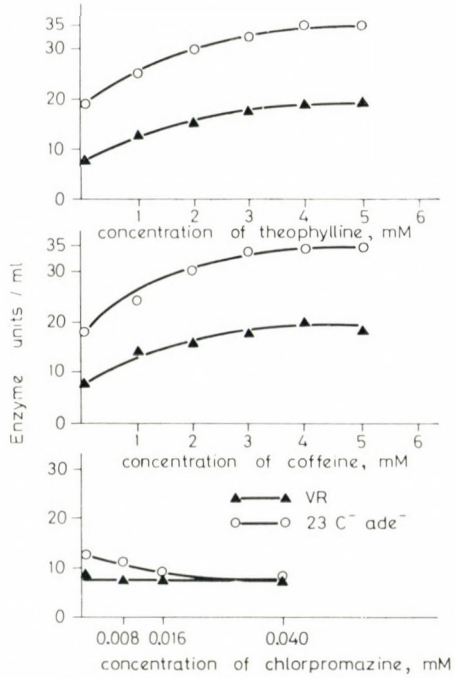

Fig. 6. Changes induced by caffeine, theophylline, and chlorpromazine in alkaline phosphatase synthesis in the prototrophic (VR) and adenine auxotrophic (23C⁻ ade⁻) strains of *B. anthracis*

phic strain of *B. anthracis* has been studied. At concentrations of 10—50 μM 2'4-dinitrophenol suppressed the growth of bacteria while it moderately enhanced the production of alkaline phosphatase (Fig. 7).

The decrease in the ATP level induced by 2'4-dinitrophenol seemed to derepress enzyme synthesis by the prototrophic strain. In order to obtain direct evidence of the regulator role of ATP, the prototrophic and adenine auxotrophic strains of *B. anthracis* were cultivated in a medium containing 1.0 mM phosphate and 50 mg/ml adenine. Samples were taken from the cultures at various phases of bacterial growth. ATP was extracted by perchloric acid and its level was determined by means of luciferase (Fig. 8).

Under the experimental conditions chosen, 50 μg/ml adenine led to a repression of enzyme synthesis in the mutant. The synthesis of ATP showed

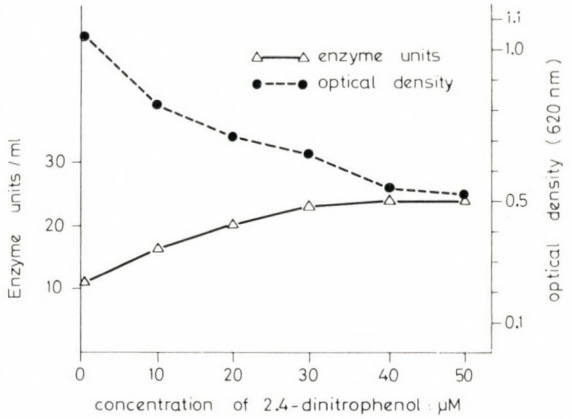

Fig. 7. Derepression of alkaline phosphatase by 2'4-dinitrophenol in the prototrophic (VR) strains of *B. anthracis*

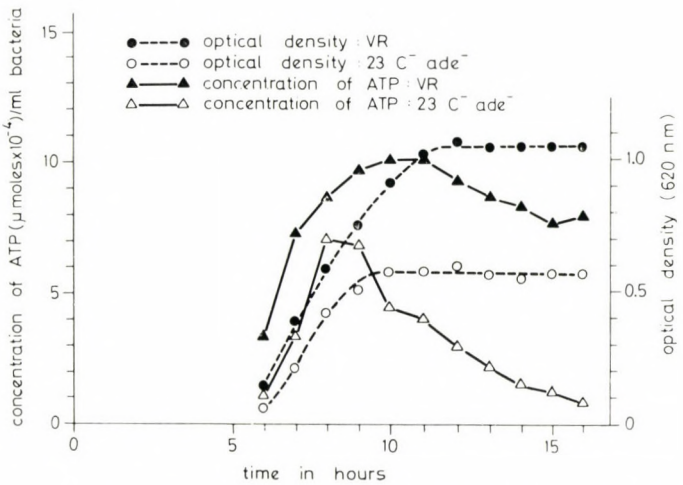

Fig. 8. Correlation between ATP synthesis and optical density in growing cultures of the prototrophic (VR) and adenine auxotrophic (23C⁻ ade⁻) strains of *B. anthracis*

an increase parallel to the growth of the bacterial cultures and exhibited a temporary stagnation before the end of the logarithmic phase. Later, ATP synthesis showed a decrease with advancing incubation time.

Discussion

A mutation occurring in a structural gene may have an indirect influence on the functions of other structural genes [15, 16]. Cyclic 3'5'-AMP plays a role in the regulation of the synthesis of various inducible enzymes [17]. Depri-

vation of the appropriate bases led to the derepression of alkaline phosphatase synthesis in the thymine, uracil and guanine dependent mutants of *E. coli* even at 2×10^{-3} M phosphate concentration. Induction is possibly associated with changes in the nucleotide pools [18]. There are at least two regulator systems involved in the regulation of alkaline phosphatase production by *B. anthracis*, both being in close connection with purine synthesis. Mutations affecting the initial steps of pyrimidine, vitamin and purine synthesis (before closing of the ring) induce no changes in enzyme production or in its repressibility. Conversion of xanthylic acid into guanylic acid is inhibited in the GMP synthetase deficient mutant. Enzyme synthesis in this mutant failed to be derepressed even at low phosphate concentrations and it became insensitive to phosphate deprivation [4]. The conversion of inosinic acid into adenylic acid is catalyzed by two enzymes. The capsulated variants of the adenylosuccinate synthetase and adenylosuccinate eyase deficient adenine dependent mutants of *B. anthracis* lost their virulence [5] and at the same time both mutations resulted in the derepression of alkaline phosphatase production [4].

The derepressed enzyme production was repressed by 1.5—1.6 mM orthophosphate in the adenylosuccinate lyase deficient mutant. The same rate of repression was caused by 50 μg/ml adenine at 0.5 mM phosphate concentration. Adenosine resulted in a partial repression even at the concentration of 100 μg/ml while inosine had no effect on the mutant's derepressed enzyme production. The low adenine concentration (10 μg/ml) used in part of the experiments failed to support the synthesis of adenine nucleotides of co-repressor effect, while a higher amount of adenine allowed the formation of appropriate amount of co-repressor. The repressor effect of cyclic-AMP on alkaline phosphatase synthesis in *E. coli* is well known [17]. The adenine nucleotides including cyclic-AMP repressed enzyme production by the adenine auxotrophic strain of *B. anthracis* and at the same time failed to influence enzyme synthesis by the prototrophic strain. Caffeine and theophylline inhibiting cyclic-AMP phosphodiesterase derepressed to a certain extent enzyme synthesis in both the auxotrophic and the prototrophic strains. In contrast with the enzyme of mammalian cells, *E. coli* phosphodiesterase was not inhibited by methylxanthines [19]. Cyclic-AMP could not be detected in either the prototrophic strain or the adenine dependent mutant of *B. anthracis*. This finding was supported by the absence of both adenylcyclase and cyclic-AMP phosphodiesterase, in other members of the genus *Bacillus viz.*, in *B. cereus*, *B. subtilis*, and *B. pumilus* [20]. The antibacterial effect of chlorpromazine used as enzyme inhibitor in our experiments probably manifests itself on the cellular membrane [21] and through the SH-enzymes [22].

The finding that adenine nucleotides repressed alkaline phosphatase production by the mutant 2'4-dinitrophenol derepressed enzyme synthesis of the prototrophic strain, raised the possibility that adenine nucleotides may play

a role in regulation as co-repressors. The fact that the adenine nucleotides studied were unable to induce a complete repression of enzyme synthesis could be explained by their poor ability to penetrate and by the wide substrate spectrum of alkaline phosphatase. The substrate specificity of alkaline phosphatase for p-nitrophenyl phosphate and adenylic acid was practically identical in *B. anthracis*, *B. cereus*, *B. megaterium* and *B. subtilis* [23]. The repressor effect of inosine monophosphate on enzyme production by the mutant became effective only above the concentration of 0.5 mM which might be in connection with the phosphate torn off from IMP. IMP at the concentrations applied exerted no effect on enzyme synthesis by the prototroph. This was in contrast with the possibility that the accumulation in the mutant of an intermediary, *e.g.* of IMP, would result in the derepressed synthesis of alkaline phosphatase.

When compared to that in the prototroph, the rate of ATP synthesis decreased in the adenine dependent mutant, supporting the physiological role played by adenine nucleotides as co-repressors in the alkaline phosphatase production of *B. anthracis*. The role of the adenine nucleotide pool may not be of universal validity. Enzyme synthesis in the adenylosuccinate lyase deficient mutant of *B. subtilis* failed to become derepressed even at low phosphate concentrations [24]. The latter finding suggests that the relation between gene loci regulating the production of alkaline phosphatase and certain structural genes involved in purine synthesis varies in different species of bacteria. The amount of adenine nucleotides synthesized in the mutants may be influenced by the reduced capacity of the biosynthetic pathways still functioning after the mutation, and by the presence of catabolic enzymes affecting the intermediates of purine synthesis in the cells.

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INHERITANCE OF SUSCEPTIBILITY AND RESISTANCE TO RAUSCHER LEUKAEMIA VIRUS

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Summary. Susceptibility to Rauscher leukaemia virus is determined by two genes, *viz.* Rv-1 and Rv-2. Locus Rv-1 and Rv-2 determines the susceptibility to LLV and SFFV, respectively. On the Rv-1 locus resistance, on the Rv-2 locus susceptibility, is dominant. The two genes segregate independently of each other. In Rv-$2^{S,S}$ and Rv-$2^{S/r}$ mice the degree of tumour-specific antibody production is determined by the Rv-1 locus. In case of genotype Rv-$1^{R/R}$ Rv-$2^{S/r}$, interferon production may be a factor determining resistance.

The genetic determinants of the susceptibility to mouse leukaemia viruses have been investigated in Gross [1] and in Friend [2] leukaemia, the former being a lymphoid, the latter an erythroid leukaemia. The susceptibility to Friend virus is determined by two gene loci, *viz.* Fv-1 and Fv-2 [2]. Among the two components of the Friend virus complex, the activity of SFFV (spleen-focus-forming virus, *i.e.* the virus responsible for erythroblastic transformation) depends on the Fv-2 gene, whereas that of the LLV (lymphoid leukaemia virus) on the Fv-1 gene. On locus Fv-1 resistance, on locus Fv-2 susceptibility is dominant. The effect of the Fv-1 locus manifests itself only in genotype Fv-$2^{S/S}$ of Fv-$2^{S/r}$ because in the case of genotype Fv-$2^{r/r}$, erythroblastic transformation does not occur. Genes Fv-1 and Fv-2 segregate independently of each other.

The Rauscher virus is another complex virus inducing erythroleukaemia [3, 4]. The aim of the present study was to explore (i) whether the two-gene hypothesis can be applied for Rauscher leukaemia; and (ii) whether there is any connexion between these gene loci and the degree of tumour-specific antibody production. As regards the latter question, some early results have already been published [5]. Inbred mice were infected with large doses of virus to exclude the possible effect of the H-2 locus on the course of the illness [6].

Materials and methods

Virus. Rauscher virus was obtained from the enlarged spleens of leukaemic Balb/c mice. The spleens, removed in the 3rd week after inoculation, were homogenized in phosphate buffer under sterile conditions to prepare a 20% suspension. This was centrifuged at 5000 *g* at 4°C for 15 min. The supernatant was stored at —70°C until used. Animals were intraperitoneally infected with 0.2 ml of this suspension.

Mice. Inbred eight-week-old Balb/c, DBA/1 and C57Bl/10Sn mice and their F1 and F2 hybrids were used, F_1 hybrids were obtained by reciprocal crossings.

Course of illness. The longest observation period was 360 days after infection. Splenomegaly was examined weekly by palpation, and by weighing the spleen after death. According to the dynamics of spleen weight, three basic types of illness were distinguished, *viz.* (I) early death following constant increase of spleen weight (progressive); (II) initial splenomegaly followed by partial or complete remission, exacerbation and late death (biphasic); (III) slight splenomegaly followed by complete remission (resistant).

Results

Fig. 1 shows spleen weight for Balb/c, DBA/1 and C57Bl/10Sn mice infected by Rauscher virus. Splenomegaly is expressed by the spleen index.

The characteristic course of illness was progressive in Balb/c mice and biphasic in DBA/1 mice. C57Bl/10Sn mice proved to be resistant. The course was independent of sex in each strain.

The F1 hybrids, due to the reciprocal crossings, were divided into six groups each consisting of 123 to 147 mice.

The (Balb/c × C57Bl/10Sn) F1 mice proved susceptible to Rauscher virus; they showed biphasic leukaemia. By the 360th day, 89% of the infected mice had died. Mortality reached the 50% level on the 200th day (Fig. 2).

The spleen index reached 4 to 6 between days 10 and 15. This was followed by progression in 2% of the animals. The others showed a remission. The percentages of the animals whose spleen index was 4 or higher at the indicated times after infection are shown in Fig. 3. The tendency of the course is clear in spite of individual differences in the degree and duration of remission.

The (DBA/1 × Balb/c) F1 hybrids showed the biphasic course characteristic of the DBA/1 mice; the degree of splenomegaly on the other hand was similar to that observed in the Balb/c mice. The mortality rate was 100%; about 90% of the mice died in the phase of exacerbation.

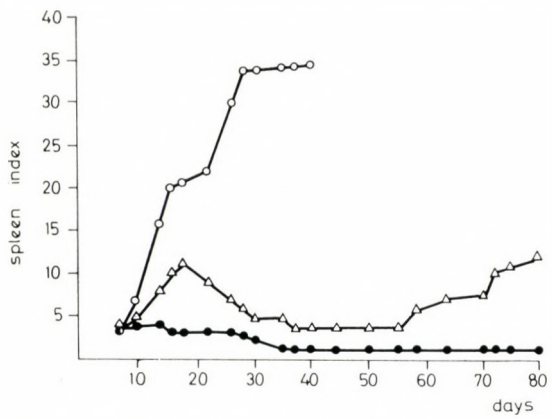

Fig. 1. Dynamics of spleen weight in Balb/c, DBA/1 and C57Bl/10Sn mice after Rauscher virus infection. ○ — ○ = Balb/c, △ — △ = DBA/1, ● — ● = C57Bl/10Sn. Spleen index = mean spleen weight for infected animals vs. that for noninfected control animals

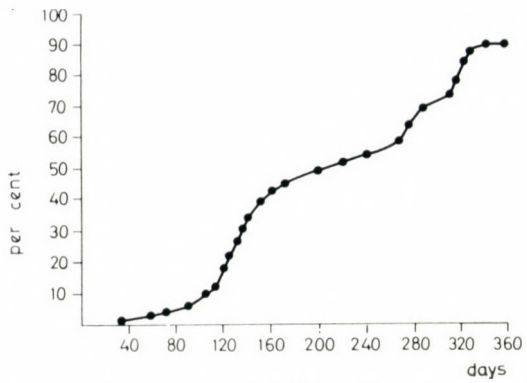

Fig. 2. Cumulative mortality of (Balb/c × C57B1/10Sn) F1 mice infected with Rauscher virus as a function of time

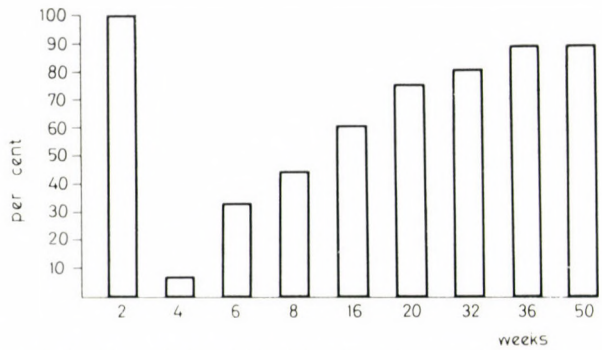

Fig. 3. Percentage of (Balb/c × C57B1/10Sn) F1 mice showing significant splenomegaly in different weeks after inoculation with Rauscher virus

The (DBA/1 × C57B1/10Sn) F1 hybrids proved to be as resistant as the C57B1/10Sn parent strain did. In this group no specific mortality had occurred by the 360th day.

The course of illness in the (DBA/1 × Balb/c) F1 and (DBA/1 × C57B1/10 Sn) F1 hybrids is illustrated in Fig. 4. The susceptibility (or resistance) of the F1 hybrids was independent of the direction of the crossing.

Each group of F2 generations contained 138 to 147 mice. Data for the (Balb/c × C57B1/10Sn) F2 hybrids are summarized in Table I.

The illness was progressive in 20% of the animals (mean survival, 53 days). A biphasic course was observed in 54%. These mice died after a remission of variable duration between postinfection days 121 and 334; 25% of the animals proved to be resistant.

In (DBA/1 × Balb/c) F2 hybrids two different types of course were observed (Table II).

From the 3 : 1 ratio of biphasic to progressive cases it follows that the course characteristic of the DBA/1 strain was inherited dominantly.

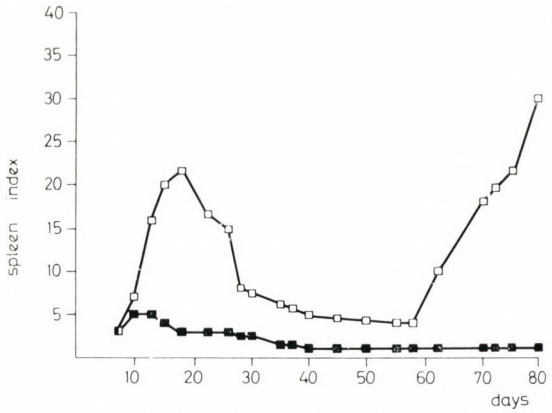

Fig. 4. Dynamics of spleen weight in (DBA/1×Balb/c) and (DBA/1×C57B1/10Sn) F1 mice after infection with Rauscher virus. □ — □ = (DBA/1×Balb/c) F1; ■ — ■ = (DBA/1 × ×C57B1/10Sn) F1

Table I

Distribution by type of course of Rauscher leukaemia in (Balb/c × C57B1/10Sn) F2 mice

Type of illness	Distribution in	
	absolute value	per cent
Progressive	28/138	20.3
Biphasic	75/138	54.3
Resistant	35/138	25.4

Table II

Distribution by type of course of Rauscher leukaemia in (DBA/1 × Balb/c) F2 mice

Type of illness	Distribution in	
	absolute value	per cent
Progressive	40/145	28.6
Biphasic	105/145	72.4
Resistant	0/145	0.0

Table III

Distribution by type of course of Rauscher leukaemia in (DBA/1 × C57B1/10Sn) F2 mice

Type of illness	Distribution in	
	absolute value	per cent
Progressive	0/147	0.0
Biphasic	72/147	49.0
Resistant	75/147	51.0

Results for the (DBA/1×C57Bl/10Sn) F2 hybrids are summarized in Table III. Of these animals 51% proved to be resistant, the remainder showed a slight splenomegaly characteristic of the C57Bl/10Sn strain, and that was followed by a remission and a slowly developing splenomegaly. The mice died between the 63rd and 306th days.

Discussion

Mouse strains Balb/c, DBA/1 and C57Bl/10Sn can be characterized as susceptible, moderately susceptible and resistant, respectively, to Rauscher leukaemia virus. Supposing that this susceptibilitiy to Rauscher virus is controlled by the two gene loci Rv-1 and Rv-2, the three mouse strains may be characterized as follows.

Balb/c : Rv-$1^{s/s}$ Rv-$2^{S/S}$

DBA/1 : Rv-$1^{R/R}$ Rv-$2^{S/S}$

C57Bl/10Sn : Rv-$1^{R/R}$ Rv-$2^{r/r}$

The results obtained with the F1 and F2 hybrids (Balb/c×C57Bl/10Sn) and (DBA/1×Balb/c) have confirmed this supposition. The moderate susceptibility of the (Balb/c×C57Bl/10Sn) F1 and (DBA/1×Balb/c) F1 hybrids corresponds to the Rv-$1^{R/s}$ Rv-$2^{S/r}$ and Rv-$1^{R/s}$ Rv-$2^{S/S}$ genetical constitution, respectively.

Table IV

Distribution of Rv-1 and Rv-2 genotypes in (Balb/c × C57Bl/10Sn) F2 mice

♀ \ ♂	$1^R\ 2^r$	$1^s\ 2^r$	$1^R\ 2^S$	$1^s\ 2^S$
$1^R\ 2^r$	$1^{R/R}\ 2^{r/r}$	$1^{R/s}\ 2^{r/r}$	$1^{R/R}\ 2^{S/r}$	$1^{R/s}\ 2^{S/r}$
$1^s\ 2^r$	$1^{R/s}\ 2^{r/r}$	$1^{s/s}\ 2^{r/r}$	$1^{R/s}\ 2^{S/r}$	$1^{s/s}\ 2^{S/r}$
$1^R\ 2^S$	$1^{R/R}\ 2^{S/r}$	$1^{R(s}\ 2^{S/r}$	$1^{R/R}\ 2^{S/S}$	$1^{R/s}\ 2^{S/S}$
$1^s\ 2^S$	$1^{R/s}\ 2^{S/r}$	$1^{s/s}\ 2^{S/r}$	$1^{R/s}\ 2^{S/S}$	$1^{s/s}\ 2^{S/S}$

Table IV shows the combinations expectable for the F2 generation of (Balb/c×C57Bl/10Sn) mice if it is assumed that Rv-1 and Rv-2 segregate independently of each other. Supposing that on the gene locus Rv-1 resistance, and on the locus Rv-2 susceptibility, is dominant, mice of genotypes Rv-$1^{s/s}$ Rv-$2^{S/S}$ and Rv-$1^{s/s}$ Rv-$2^{S/r}$ (*i.e.* 19% of the mice) should be susceptible, those of genotypes Rv-$1^{R/R}$ Rv-$2^{S/r}$, Rv-$1^{R/s}$ Rv-$2^{S/r}$, Rv-$1^{R/R}$ Rv-$2^{S/S}$ and Rv-$1^{R/s}$ Rv-$2^{S/S}$, (*i.e.* 56%) should be moderately susceptible and those of genotypes Rv-$1^{R/R}$ Rv-$2^{r/r}$, Rv-$1^{R/s}$ Rv-$2^{r/r}$ and Rv-$1^{s/s}$ Rv-$2^{r/r}$ (*i.e.* 25%) should be

resistant. The percentage values 20%, 54% and 25% are consistent with the calculated distribution. Furthermore, the present results have proved that the Rv-$1^{s/s}$ Rv-$2^{r/r}$ mice are resistant to LLV. It has been shown [4, 11] that to develop leukaemia, Rv-$1^{s/s}$ mice must be infected with LLV before reaching four weeks of age.

The expectable genotypes of the generation (DBA/1 × Balb/c) F2 are

Table V

Distribution of Rv-1 and Rv-2 genotypes in (DBA/1 × Balb/c) F2 mice

♀ \ ♂	$1^R\ 2^S$	$1^S\ 2^S$
$1^R\ 2^S$	$1^{R/R}\ 2^{S/S}$	$1^{R/s}\ 2^{S/S}$
$1^s\ 2^S$	$1^{R/s}\ 2^{S/S}$	$1^{s/s}\ 2^{S/S}$

shown in Table V. Since all individuals in this generation are Rv-$2^{S/S}$ homozygotes, the occurrence of 3 biphasic cases for each progressive case can be attributed to a 3 : 1 ratio of the occurrence of Rv-$1^{R/R}$ + Rv-$1^{R/s}$ to that of Rv-$1^{s/s}$.

The results obtained for the (DBA/1 × C57Bl/10Sn) F1 and F2 mice are inconsistent with this scheme, *viz.*, the 100% resistance of the F1 hybrids disagrees with the Rv-$1^{R/R}$ Rv-$2^{S/r}$ genotype. Similarly, the 50% resistance in the F2 generation (Table III) is significantly different from the calculated 25%. The expectable genotypes of the (C57Bl/10Sn) F2 mice as calculated on the basis of the susceptibility of the parent strains are shown in Table VI.

Table VI

Distribution of Rv-1 and Rv-2 genotypes in (DBA/1 × C57Bl/10Sn) F2 mice

♀ \ ♂	$1^R\ 2^S$	$1^R\ 2^r$
$1^R\ 2^S$	$1^{R/R}\ 2^{S/S}$	$1^{R/R}\ 2^{S/r}$
$1^R\ 2^r$	$1^{R/R}\ 2^{S/r}$	$1^{R/R}\ 2^{r/r}$

Obviously, mice of genotype Rv-$1^{R/R}$ Rv-$2^{r/r}$ are resistant, whereas those of genotype Rv-$1^{R/R}$ Rv-$2^{S/S}$ are moderately resistant. Genotype Rv-$1^{R/R}$ Rv-$2^{S/r}$, which has been calculated to occur in 50%, should manifest itself in a moderate susceptibility. The shift toward higher resistance is suggestive of the role of further gene(s). In this relation it may be noted that Rauscher virus infection is followed in DBA/1 mice by an intensive interferon

production [7], which is apparent in (DBA/1 × C57Bl/10Sn) F1 hybrids too, but does not ensue in (DBA/1 × Balb/c) F1 mice [8]. Accordingly, the degree of interferon production might be a resistance-determining factor in the case of genotype Rv-$1^{R/R}$ Rv-$2^{S/r}$. The protective effect of interferon might manifest itself *via* an inhibition of cell division [9] and/or an enhancement of phagocytosis [10]. If the intensive interferon production in Rauscher virus-infected mice were determined by a dominant gene, then the proportion of resistant individuals in the (DBA/1 × C57Bl/10Sn) F2 generation should be calculated at 63%. The 51% level observed by us is suggestive of a complexity of the genetical factors governing the degree of interferon response. The uniformity of the response of the F1 generation may be explained by the high-degree inbred nature of the parent strains.

Among the two components of the murine erythroleukaemia viruses, the immunosuppressive effect of SFFV is more intensive than that of LLV [11]. On the other hand, it is LLV that enables SFFV to replicate in cells of the immune apparatus and thus significantly to accelerate the progression of leukaemia. Accordingly, in mice of Rv-$2^{S/S}$ or Rv-$2^{S/r}$ genotype the degree of the immune response and, consequently the course of the illness might be controlled by the Rv-1 locus. The delayed course characteristic of genotypes Rv-$1^{R/S}$ and Rv-$1^{R/R}$ as well as the intensive tumour-specific antibody production by DBA/1 and C57Bl/10Sn mice, both being of the Rv-$1^{R/R}$ genotype, is consistent with this view.

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AETIOLOGICAL SIGNIFICANCE OF VIRUSES AND ROLE OF LYMPHOCYTES IN CERTAIN GASTROINTESTINAL DISEASES

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Summary. About 10 000 preparations obtained from nearly 500 patients and healthy controls were studied. Cells of scrapings of oral ulcers and lymphocytes were examined with immunofluorescence and the lymphocyte transformation test as well as with the rapid swelling reaction of lymphocytes under viral effect. Adenovirus and herpesvirus antigens were significantly more often detected in the lymphocytes of aphthous patients than in the controls. The simultaneous presence of the two virus types was frequently observed in both cell types of the same patient. About two-thirds of the patients gave positive lymphocyte transformation reaction with type 1 adenovirus and half of them with herpesvirus. A similar incidence was found with the rapid lymphocyte swelling reaction. Patients with ulcerative colitis displayed 36.4% positivity in the lymphocyte transformation test with the latent adenovirus types 1, 2, 5 and 6 while with herpesvirus this rate was 17% in patients with hepatic cirrhosis and 20% in diabetic patients, in contrast to the 6—8% positivity of patients with other diseases and healthy persons, which refers to a sensitivity of the cells to those viruses. Thus, the close interaction between lymphoid cells and latent adenovirus types or herpesvirus appears to play a direct or indirect role, perhaps as a part of allergic factor, in the pathogenesis of some gastrointestinal diseases.

Adenoviruses may play an important role in some diseases of the alimentary tract and of the abdominal organs. These diseases are usually associated with respiratory symptoms [1—3]. Several adenovirus strains have been isolated in our previous experiments from different excretions of children who had been operated for appendicitis and suffered at the same time from mesenteric lymphadenitis and pharyngitis. The significant rise in antibody titre against the isolated viruses in the convalescent sera indicated that adenoviruses too might have a role in this presumably polyaetiologic disease. According to our earlier experiments, specific adenovirus and herpesvirus antigens may be present in the cells of local lesions or in the circulating lymphocytes, and that the latter might be sensitized against the corresponding virus antigens [4].

Results of observations on patients suffering from gastrointestinal diseases will be discussed in the present paper.

Materials and methods

The adenoviruses and herpesviruses used in these experiments were partly laboratory strains, and partly strains freshly isolated from patients.

Cell cultures. Maintenance and infection of HeLa, HEp-2, MAS-A and primary human amnion cell cultures have been described earlier [5—6].

Immunofluorescence technique. Direct immunofluorescence technique was performed according to COONS and KAPLAN [7] with certain modifications [8, 9]. Circulating lymphocytes of patients were tested with fluorescein-isothiocyanate conjugated adenovirus and herpesvirus antisera produced in rabbits. Cytological preparations taken with a scalpel from the oral mucosa of patients were studied with indirect immunofluorescence technique to demonstrate herpesvirus and adenovirus antigens [10]. The direct immunofluorescence method was applied to study primary human amnion cell cultures infected with adenoviruses and herpesviruses. Adenovirus antiserum was conjugated with fluorescein isothiocyanate and herpesvirus antiserum with Rhodamine [11]. Staining was performed either simultaneously or in successive order.

Lymphocyte transformation test (LTT) was performed according to HALPERN *et al.* [12] as described earlier [13]. To study the interaction between lymphocytes and adeno or herpesviruses a cell suspension was mixed with virus and one drop was placed on a slide, covered with a coverslip and sealed with paraffin. The morphological changes were observed with the light microscope after 15, 40 and 80 min as well as after 2 and 24 hr. Lymphocyte and other cell suspensions without antigens or treated with other antigens and materials served as control.

Results

Demonstration of virus antigens from aphtha scrapings. In previous experiments, type 1 adenovirus was isolated from two patients with oral ulcers. Adenovirus antigens were demonstrated by means of immunofluorescence in the cells of patients from whom virus could not be isolated [10]. Six hundred smears of a total of 150 persons were studied with indirect immunofluorescence technique, applying adenovirus and herpesvirus antisera. Results are presented

Table I

Immunofluorescence of cells of aphtha scrapings with adenovirus and herpesvirus antisera

Total No. of cases 150

Disease	No. of cases	No. of cases positive with			No. of negative cases
		adenovirus antiserum	herpesvirus antiserum	adeno and herpesvirus antisera	
Recurrent aphtha	85	56	16	7	20
Herpes	21	5	9	3	10
Healthy control	44	1	1	0	42

in Table I. Out of 85 aphthous patients the cells of 49 revealed adenovirus-specific fluorescence, samples of 7 patients were positive with both adenovirus and herpesvirus antisera, and 9 reacted only with herpesvirus antiserum. No specific fluorescence was detected in the aphtha scrapings of 20 persons. Persons whose oral mucosa was intact and had never suffered of aphthae, were chosen for control. The scrapings were taken from their inner labial mucosa where aphthae occur frequently. A few cases of herpetic gingivostomatitis and labial herpes were also included in the examinations. Forty-two out of 44 healthy persons possessed negative cells, 1 was positive with adenovirus antiserum and 1 with herpesvirus antiserum. Neither adenovirus nor herpesvirus

antiserum produced specific fluorescence in 10 of the herpetic controls. Scrapings of the herpetic erosions showed in 6 cases specific fluorescence with herpesvirus antiserum, in 3 cases with both antisera and in 2 cases with adenovirus antiserum.

Demonstration of virus antigens in the circulating lymphocytes of aphthous patients. Certain viruses such as cytomegalovirus, Epstein-Barr virus, etc. may be present in circulating human lymphocytes without ever having caused recognizable symptoms [14, 15]. Considering the fact that the cellular infiltration of aphthous ulcers consists of lymphocytes [16], it was attempted to establish whether the virus antigens found in the aphtha scrapings were present also in the lymphocytes of aphthous and control individuals. Seventy-six adults were studied with direct immunofluorescence technique. Data are presented in Table II. As shown, patients with multiform erythema were all herpes positive.

Table II

Immunofluorescence of circulating lymphocytes with adenovirus and herpesvirus antisera

Total No. of cases 76

Disease	No. of cases	No. of cases positive with			No. of negative cases
		adenovirus antiserum	herpesvirus antiserum	adeno and herpesvirus antisera	
Recurrent aphtha	43	27	30	22	8
Multiform erythema	6	2	6	2	0
Recurrent labial herpes	6	3	5	2	0
Oral lichen	4	2	3	1	0
Oral cancer	3	1	1	1	2
Leukoplakia	2	2	2	2	0
Xerostomia	2	0	2	0	0
Healthy oral mucosa	10	0	1	0	9

The same was the case with the patients with recurrent labial herpes, with a single exception, while about two-thirds of the patients with recurrent aphtha were adenovirus and herpesvirus positive (Fig. 1).

Studying oral lichen, oral cancer, leukoplakia and xerostomia cases as control, it was found that nearly half of them was adenovirus positive and over two-thirds herpesvirus positive. The lymphocytes of only 1 of the 10 persons with intact mucosa revealed herpesvirus positivity.

When the opportunity was given to repeat the examinations in the same patients every 3—4 weeks, it was found that more lymphocytes showing specific fluorescence were present before than after the relapse.

Patients whose cells contained both virus antigens simultaneously, were detected in aphtha cell examinations as well as in lymphocyte studies, though more frequently in the latter case.

Experimental double infection was carried out with type 1 adenovirus and herpesvirus isolated in our laboratory. Primary human amnion cells propagated on coverslip fragments in test tubes, were infected with the viruses. Double immunofluorescence method was applied with fluorescein isothiocyanate conjugated adenovirus antiserum and Rhodamine conjugated herpesvirus antiserum.

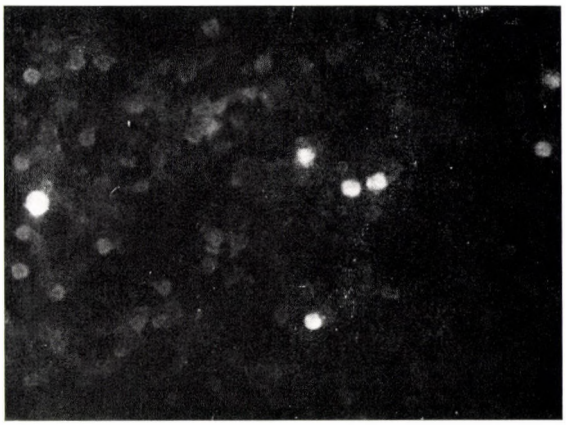

Fig. 1. Lymphocytes showing specific fluorescence with conjugated type 1 adenovirus antiserum

A yellowish-green fluorescence characteristic of adenovirus antigen and conjugate was found in the cell nucleus of adenovirus infected cultures. An orange-red fluorescence characterizing the herpesvirus antigen and conjugate, was detected first in the nucleus and later in the cytoplasm of herpesvirus infected cells. To obtain fluorescence of identical intensity, adenovirus infection had to precede that of the herpesvirus by 48 hours, when a 100 c.p.u. dose was applied. Under these conditions the adenovirus infection did not inhibit the development of herpesvirus antigen. Both types of specific fluorescence could be observed in doubly infected cell cultures. Certain cells of the preparations displayed only adenovirus fluorescence while others only herpesvirus fluorescence and in certain cells both virus antigens were detectable. In the case of double infection, the yellowish-green adenovirus fluorescence was situated in the nucleus, while the orange-red herpesvirus fluorescence in the cytoplasm or near the nuclear membrane, often as small granules [11].

Lymphocyte transformation studies in aphthous patients. Lymphocyte cultures of 89 adults were studied; 45 were aphthous patients and 44 controls. The rate of blastic transformation is presented in Table III according to the

Table III

Lymphocyte transformation test in patients with recurrent aphthae using adenovirus and herpesvirus antigens

	Adenovirus types							HSV	Per cent of transformed cells	Adenovirus types							HSV	
	1	3	5	7	8	12	18			1	3	5	7	8	12	18		
No. of	14	39	37	39	44	39	38	22	0— 5	39	42	40	40	44	43	40	41	No. of
cases	16	6	8	6	1	5	3	15	5—10	4	2	2	4		1	2	3	cases
	11					1	3	8	10—15	1		2				1		
	3						1		15—20							1		
	1								20—25									

No. of aphthous patients, 45 No. of healthy controls, 44

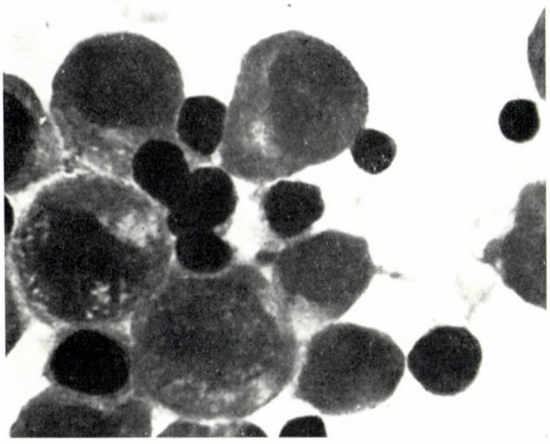

Fig. 2. Lymphoblasts developing upon the effect of type 1 adenovirus in lymphocyte cultures of patients with recurrent aphthae

antigens and the number of persons examined. It is seen that the lymphocytes of patients with recurrent aphtha revealed a higher rate of blastic transformation (Fig. 2) with type 1 adenovirus and herpesvirus antigens than the controls. The number of positive cases in which the rate of lymphocytes transforming with the two virus types was over 5%, proved to be significantly higher in the aphthous group. Applying other adenovirus antigens, no such quantitative differences were found between aphthous patients and controls. This shows that it is not the common group-specific adenovirus antigen that induces the lymphoblastic transformation.

Lymphocyte cultures which did not contain antigen showed about 1% blastic transformation in both aphthous and control persons. Phytohaemagglu-

tinin (PHA) induced in both groups 30 to 70% blastic transformation, while control cultures containing tissue antigens, showed a 0.5 to 2% transformation rate.

Certain adenovirus types stimulated changes other than blastic transformation in lymphocytes, *e.g.* type 8 adenovirus induced the appearance of mono- and multinuclear plasma cells, while the highly oncogenic type 18 effected multinuclear giant cells.

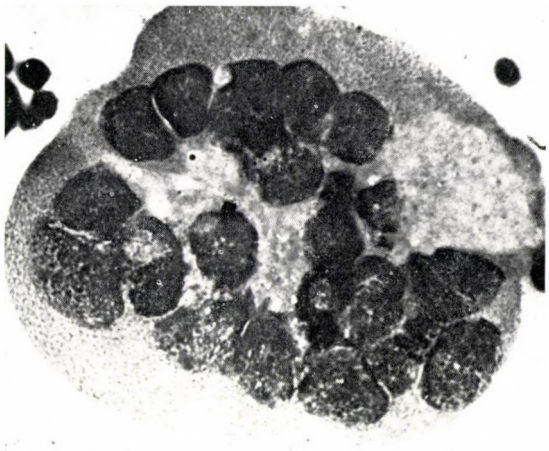

Fig. 3. Multinuclear giant cell developing upon the effect of type 18 adenovirus

Lymphocyte transformation in patients suffering from diseases of the digestive tract. The above results indicated that type 1 adenovirus and herpesvirus antigens were present in a significantly higher per cent in the lymphocytes of aphthous patients than of the controls, furthermore that the rate of blastic transformation was also considerably higher in the lymphocytes of these patients. It has been observed that not only lymphoid tissue but also theliolymphocytes might be found under the mucosal epithelium, among the cells of the alimentary canal [17]. The association of diseases of the digestive tract, *e.g.* ulcerative colitis with aphthous stomatitis, is well known [18]. All these had drawn our attention to the question of whether the virus types mentioned might be present in the lymphocytes of patients suffering of inflammatory or other diseases of the lower digestive tract and we wished to obtain information also on the reaction of these lymphocytes with the given virus antigens in the lymphocyte transformation test (LTT) [19].

Results are presented in Table IV according to the rate of blastic transformation. Though the number of cases studied failed to permit statistical evaluation according to diagnosis, the results provided remarkable data, if the

high number of controls is taken into consideration. Thus, the lymphocytes of patients with ulcerative colitis were more sensitive to adenovirus antigens, especially to the latent types 1, 2, 5 and 6, than were the controls. The lymphocytes of about one-third of the patients were found to be sensitized also against herpesvirus. The lymphocytes of patients with hepatic cirrhosis as well as of the diabetic patients showed an increased sensitivity to adenoviruses and to a certain extent to herpesvirus.

Table V allows a percentual comparison. It includes the summarized results of the LTT of the individual patients and 10 virus antigen types according to positive (over 5% blastic transformation) and negative (under 5%)

Table IV

Lymphocyte transformation in patients with diseases of the alimentary tract under the effect of adenovirus and herpesvirus antigens

Total No. of cases 111

Disease	No. of cases	Blastic transformation over 5%										
		Virus antigens										
		Adenovirus types										
		Latent				Epidemic			Oncogenic			
		1	2	5	6	7	8	10	12	18	HSV	PHA
Ulcerative colitis	14	9	7	8	7	2	3	3	4	3	5	14
Cancer	15	1	1		2	2			3	1	2	10
Hepatic cirrhosis	10		1	1	3	2	1	1	2	1	5	10
Controls												
Diabetes mellitus	10	3	3	1	2			1	3		7	10
Other diseases	18	4	3		1	1	1		2	1		16
Healthy persons	44	4	1	1	1	4			5	8	4	44

Disease	No. of cases	Blastic transformation under 5%										
		Virus antigens										
		Adenovirus types										
		Latent				Epidemic			Oncogenic			
		1	2	5	6	7	8	10	12	18	HSV	PHA
Ulcerative colitis	14	5	7	6	7	12	11	11	10	11	9	
Cancer	15	14	14	15	13	13	15	15	12	14	13	5
Hepatic cirrhosis	10	19	9	9	7	8	9	9	8	9	5	
Controls												
Diabetes mellitus	10	7	7	9	8	10	10	9	7	10	3	
Other diseases	18	14	15	18	17	17	17	18	16	17	18	2
Healthy persons	44	40	43	43	43	40	44	44	39	36	40	

results. Accordingly, the LTT was positive in 28 out of 440 experiments performed with lymphocytes of the healthy persons (approx. 6%). A similarly low positivity rate was found among patients suffering from other diseases or from malignant tumours of the digestive tract. No change in LTT was found in a few cases of gastric ulcer and gastritis (not presented in the Table). The 6 to 8% positivity in the controls made the 17% positivity in hepatic cirrhosis and 20% in diabetes mellitus rather striking. The 36.4% positivity in ulcerative colitis was all the more remarkable, as more than 60% of the positive cases were induced by the 4 latent adenovirus types.

Table V

Results of lymphocyte transformation test

Disease	No. of cases	No. of examinations	Blastic transformation over 5% (positive)	Blastic transformation under 5% (negative)
Ulcerative colitis	14	140	51 (36.4%)	88 (62.9%)
Cancer	15	150	12 (8.0%)	138 (92.0%)
Hepatic cirrhosis	10	100	17 (17.0%)	82 (82.0%)
Controls				
Diabetes mellitus	10	100	20 (20.0%)	80 (80.0%)
Other diseases	18	180	13 (7.2%)	167 (92.8%)
Healthy persons	44	440	28 (6.3%)	412 (93.7%)
Total	111	1110		

Table VI shows the LTT results obtained in ulcerative colitis patients. It indicates separately the percentage of blastic transformation according to the individual patients and antigens. The fact that 5% or higher blastic transformation occurred only in about 6% of the healthy persons and that the rate of transformation was only 0.1 to 1% in the lymphocyte population, made it remarkable that in ulcerative colitis the number of positive cases surpassed 50% even if only a rate of 3% or more was considered positive. This referred to a close connection between ulcerative colitis and herpesvirus or adenovirus, especially of the latent types. Referring the 3% or higher blastic transformation rate to the 56 experiments performed with the 4 latent types, the 46 positive cases meant an 80% positivity. Thus, in accordance with the data of Table VI, the lymphocytes of each ulcerative colitis patient gave a positive LTT with one or more latent adenovirus types.

The lymphocytes of the ulcerative colitis patients displayed not only the usual lymphoblastic transformation under the effect of certain virus antigens,

Table VI

Lymphocyte transformation in patients with ulcerative colitis

Designation of patients	Antigens										
	Virus antigens									HSV	PHA
	Adenovirus types										
	Latent				Epidemic			Oncogenic			
	1	2	5	6	7	8	10	12	18		
1	7*	3	5.2	5.8	0.7	1	0.3	0.5	0.4	0.8	30
2	1.5	5	5.8	5.5	5.5	1.2	1.2	2.5	3.1	5.1	50
3	3	1.5	3.6	0.6	1	1.3	2	1.5	3.5	1.6	62
4	5.8	1.8	2.5	2.3	2.6	0.3	1.2	1.7	1.6	4.7	55
5	3	2.4	4.3	3	1	2.2	4.1	0.3	0.4	2	35
6	1.2	3.6	4.8	3.9	3.4	0.6	1.2	1.6	0.2	2.3	30
7	6	5.1	9.7	6	1.9	2.4	2.3	3.5	5.4	9.4	30
8	7.3	8.8	3.2	5.7	5.3	5.2	5.8	5.4	3	5.2	30
9	5.4	9.6	5.8	8.4	2.1	2.7	3.7	5.4	1.7	5.4	90
10	11.8	6.8	11	1.8	3.1	15.6	9	10	7.6	0.6	30
11	5.8	3	3.5	4.1	0.9	1.3	9.2	2.1	0.8	1.8	65
12	4.2	6.7	11.2	1.8	1.6	5.8	3.2	10.4	7.6	0.2	30
13	5.7	4.3	5.9	8.1	2.7	1.9	2.1	2.2	1.6	4.8	75
14	6.3	9.6	7.2	8.8	1.8	2.1	1.4	2.2	0.9	5.1	70
3% and more; total	12	11	13	10	4	3	6	5	6	7	

* = the figures indicate percentage of lymphoblasts

especially of the latent adenovirus types, but similarly to aphthous patients, large cells with loose cytoplasm or multinuclear giant cells appeared in the cultures.

To throw light upon the connection between the lymphoid cells of the patients studied and the viruses, immunofluorescence studies were performed to establish whether or not the lymphocytes of the patients were carrying virus antigens. These investigations revealed that the lymphocytes of ulcerative colitis, hepatic cirrhosis and diabetic patients were carrying antigens of the viruses to which they displayed sensitivity in the LTT.

Interaction of lymphocytes and viruses. The LTT was performed with lymphocytes incubated previously for 5 days together with the viruses. It was, however, observed that significant morphological changes developed in the lymphocytes of patients a few minutes after addition of the viruses. This phenomenon was also subjected to investigation, in patients with recurrent aphthae and other oral diseases as well as in healthy persons.

Fifteen minutes after the addition of adeno or herpesviruses, certain cells of the lymphocyte suspension started swelling, showing a changed refraction and after 40 min they doubled their size. These lymphocytes could well be differentiated from the others, the swelling continued and reached its maximum within 2 hr, and by this time the diameter of the cells had become three or

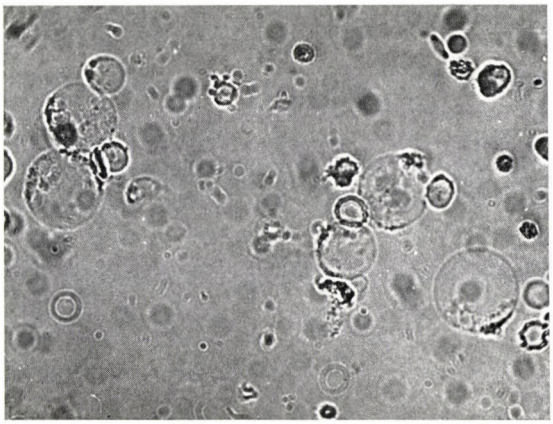

Fig. 4. Rapid swelling reaction of lymphocytes 2 hr after virus treatment

four times the original (Fig. 4). Parallel with the ballooning, the nuclear substance of the cells became pressed towards the margins. This phenomenon could be induced by both infective and inactivated viruses. No further changes occurred in the latter case, while in infective virus-treated preparations the ballooned cells decomposed generally into debris after 24 hr. As shown by Table VII, the rapid swelling reaction was observed with a few exception on the lymphocytes of aphthous patients with both adeno and herpesvirus, the rate of the ballooned cells being in most cases about 1 to 3%. Similar results were obtained in other patients and a positive swelling reaction was observed in two-thirds of the lymphocytes of apparently healthy persons, too. Thus, experiments were carried out to throw light on the specificity and mechanism of the reaction.

The ballooned cells could be removed by fibreglass filtration from the lymphocyte suspension. No ballooning was found in filtrated suspensions to which the originally applied virus had been added, while in those treated with the other virus type — *e.g.* HSV to adenovirus treated suspension — new ballooning cells appeared within 2 hr. The reaction took place equally at room temperature and 4°C, in autologous plasma as well as in Parker's 199 maintenance solution. We have attempted to activate the lymphocytes with cell extracts of human amnion and HEp-2 cultures and the cells of these suspension cultures were subjected to the activating effect of the viruses. Both control procedures gave negative results.

Discussion

The role of lymphoid cells in humoral and cellular immunity [20—22] is known. They are closely connected with the epithelial cells of the skin and mucosa. As the mucosa of the alimentary canal contains large numbers of lymphocytes [17] and comes into contact with several types of antigen such as alimentary and microbial ones, etc., their aetiological importance is assumable. When the immune apparatus of the organism is unable to control certain viruses, they enter, and persist in the lymphoid cells, where they exert a disturbing effect on the immune reaction [15].

The results suggest that, besides other factors, viruses or virus antigens and lymphoid cells may play a significant role in the development of chronic and recurrent digestive tract diseases of unknown aetiology. The presence of type 1 adenovirus and herpesvirus antigen in the oral epithelial cells and lymphocytes of a large number of patients, refers to an aetiological relationship. The simultaneous presence of adeno and herpesvirus antigens may similarly be connected with these diseases and their recurrence. Virus-induced disturbances of the immune reaction may also play a role.

According to literary data and to our own results, the lymphocyte transformation test is as valuable as the allergic skin test and, applying specific antigens, it furnishes information on the cellular immunity and sensitivity of the organism. Our investigations show that lymphocytes of patients suffering from recurrent ulcers in the alimentary tract (aphtha, ulcerative colitis) are sensitive to HSV and to the latent adenovirus types. This refers to a possible connection between these viruses or virus antigens and the said diseases. Lymphoid cells may play an important role in such connections, as the circulating lymphocytes of most patients were found to contain these virus antigens. On the basis of these facts, patients with the above-mentioned mucosal diseases may be regarded as virus carriers, who have relapses from time to time. Allergic and autoimmune factors may also play a role in the recurrences [23].

Lymphocytes of apparently healthy persons may be sensitive to different adenovirus and herpesvirus antigens as well as specific antigens of the viruses may persist in them for shorter or longer periods. This refers to the complex connection between these viruses and the lymphocytes, and one may assume that virus carrier lymphocytes play a role not only in herpesvirus or adenovirus diseases but also in other viral diseases.

The carrying of herpes and adenoviruses deserves all the more attention as recently not only certain adenovirus types but also herpesvirus type 2 have been found to cause malignant changes [24]. According to a hypothesis, confirmed also by our observations, lymphocytes become activated if the immune regulation suffers some impairment [25]. Cellular immunity appeared to be disturbed in our cases. As epithelial cells of the mucosa and lymphoid cells are

closely connected, one may assume that viruses causing periodic diseases, are released from the mononuclears into epithelial cells and induce recurrences, or may play a role in disturbances of cellular immunity. When the lymphoid cells carrying the virus or viral gene cease to control their oncogenic effect, the disease which up to then showed recurrent or chronic features, turns into a malignant disease.

Our observations on the rapid activating effect of viruses on a small percent of lymphocytes may furnish new information on the interaction between cells and viruses as well as on the function of lymphocytes. The phenomenon may also be used as a rapid diagnostic method.

Table VII

Swelling reaction of lymphocytes under the effect of adenovirus and herpesvirus antigens

Total No. of cases 65

Disease	No. of cases	Rate of swollen lymphocytes						Negative	
		0.5–1%		1–3%		over 3%			
		adeno-virus	herpes-virus	adeno-virus	herpes-virus	adeno-virus	herpes-virus	adeno-virus	herpes-virus
Recurrent aphthae	34	10	8	23	24		1	1	1
Recurrent herpes	7	1	1	6	6				
Controls									
Oral lichen	3	1		2	3				
Multiform erythema	5	1	3	4	1				1
Oral cancer	2	1			2			1	
Benign tumours	1	1			1				
Healthy persons	13	2	3	6	5		2	5	3

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MYCOBACTERIUM TUBERCULOSIS STRAINS SHOWING POSITIVE IRON UPTAKE

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Summary. Strains showing a positive iron uptake reaction have been identified to belong to *Mycobacterium tuberculosis*, a species known as not giving this reaction. The strains isolated from patients were resistant to INH, failed to produce catalase and showed a moderate pathogenicity in the guinea pig. In other characteristics they corresponded to the drug sensitive *M. tuberculosis* strain H_{37}Rv.

The capacity for iron uptake of rapidly growing mycobacteria was described by Szabó [1], confirmed by Tison *et al.* [2] and further investigated by Pattyn and van Ermengem [3]. The use of this stable characteristic has been recommended for the taxonomy of atypical mycobacteria [4].

Mycobacterium tuberculosis and *M. bovis* have been known as not reducing iron ammonium citrate. In this paper an account is given of *M. tuberculosis* isolates giving a positive reaction.

Materials and methods

Methods were as described in [4]. For amidase test Bönicke's method [5] was used. Drug resistance was tested as described by Canetti *et al.* [6], nitrate reduction by the method of Szabó and Vandra [7].

Results and discussion

For the culturing of specimens from patients receiving INH, iron salt medium allowing the growth of strains with decreased catalase activity was used. On this medium some *M. tuberculosis* strains showed brick-coloured growth. Examination of these cultures is presented in Table I.

M. tuberculosis strains with brick or rust red colour instead of the usual yellow one were resistant to INH, failed to produce catalase and showed a decreased pathogenicity to the guinea pig. In other properties they corresponded to the control strain. It has been concluded that iron uptake is associated with INH resistance and catalase negativity and the latter may be responsible for the unusual behaviour of these cultures.

Table I

Properties of the control and of iron uptake positive M. tuberculosis strains

Strain	Growth at		Niacin	Nitrate reduction	Lipase	Aryl-sulphatase	Amidase reactions*	Catalase	INH 1 μg/ml**	Virulence***
	25	37°C								
$H_{37}Rv$ (control)	—	+	+	+	±	—	3 (5.6)	+	S	++++
227	—	+	+	+	—	—	3 (5.6)	—	R	+
228	—	+	+	+	—	—	3 (5.6)	—	R	+
229	—	+	+	+	—	—	3 (5.6)	—	R	+
230	—	+	+	+	—	—	3 (5.6)	—	R	+
231	—	+	+	+	—	—	3 (5.6)	—	R	+

* Amidase reactions: 3 = urease, 5 = nicotinamidase, 6 = pyrazinamidase; bracketed figures indicate weak reactions

** S = sensitive, R = resistant

*** Virulence to guinea pigs, 0.001 mg (wet wt) bacteria injected subcutaneously into the right inguinal region; ++++ = generalized tuberculosis, + = local changes

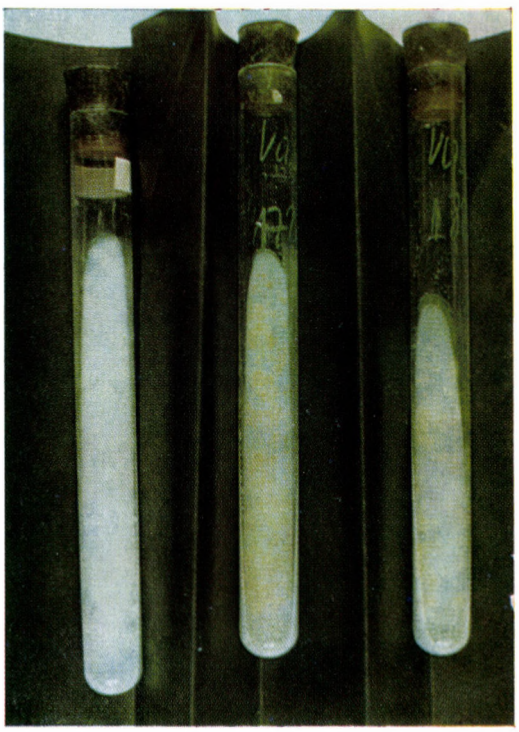

Fig. 1. Control strain *M. tuberculosis* $H_{37}Rv$ (left) and two *M. tuberculosis* strains showing positive iron uptake reaction (right) after 4 weeks incubation on Löwenstein–Jensen medium containing 2% ferric ammonium citrate

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A CRYPTIC PROPHAGE CARRIED BY BACILLUS CEREUS

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Summary. Mitomycin C (MC) added to a young culture of *Bacillus cereus* strain W induced marked lysis. The concentration of MC affected the degree of lysis as well as the concentration of phage and bacteriocin (phospholipase A) in the lysate. The factor controlling the production of bacteriocin could be eliminated by chemical agents; thus *cin*$^-$, not capable of producing bacteriocin, was obtained regularly. With the aid of *cin*$^-$ bacteria, a phage was detected in the lysate of wild-type bacteria, which was denoted as phage *wx*, controlled by a prophage. Phage *wx* is of temperate character. In addition, these lysates also contained a proportion (about 10^{-3} to 10^{-4}) of clear-plaque forming mutants *(wxc)*. Phages *wx* and β differed in their antigenic structures and other characteristics; thus they are not of common genetic origin. A striking feature of phage *wx* is its high host specificity; none of the numerous strains belonging to any species of the genus *Bacillus* were attacked except the cured derivative of strain W, denoted as *cin*$^-$ of *B. cereus*.

MCCLOY [1] described the lysogenic strain W of *Bacillus cereus*, which liberates a phage highly specific to *B. anthracis*, whereas strains belonging to other species of the genus *Bacillus* are practically all resistant. The characteristics of this phage, designated as Wβ, and its allelic mutant Wα which forms clear plaques, have been studied in several respects [2, 3]. Phage Wβ may be considered semitemperate because in certain cases it forms labile complexes with wild strains of *B. anthracis* [4]. Further mutants were also described, which can be considered to be the alleles of phage β [2, 3, 5]. The antigenic structures of the alleles were found to be identical. Phage β and its mutants were designated as phage group W.

It has recently been reported [6] that the addition of a small amount of mitomycin C (MC) to a culture of *B. cereus* strain W growing exponentially resulted in a residual growth followed by mass lysis of the bacteria. The lysate contained both phage β and a bacteriocin of narrow spectrum. The bacteriocin of strain W was considered to be phospholipase A, like that produced by certain strains of *B. megaterium* (see reviews [7, 8]) and found by OZEKI *et al.* [9] to be identical with that enzyme.

It seemed reasonable to study the *B. cereus* strain W to determine the relations between the lysogenic and bacteriocinogenic features. In the course of these studies we found that besides prophage β there exists another unknown prophage, remarkable for its extraordinary specificity.

Materials and methods

Bacterial strains. The 12 strains of *B. cereus* which can be induced by MC have already been reported [6] in connection with our detailed studies on strain W producing phospholipase A. Besides these strains we have applied a number of *B. cereus* strains as indicators. These strains were freshly isolated from soil samples and were identified on the basis of the following criteria: colony morphology, haemolysis, and behaviour on egg yolk agar. The strains applied in our work are listed in Table I.

Table I

Number and origin of the strains applied for determining the host range of phage wxc

Species	Designation	Characteristics	No. of strains	Source	Reference
B. cereus			11	Own collection	6
	wild type		28	Freshly isolated	
	T		1		
	6464		1	C. B. THORNE	10
	6464 C		1	C. B. THORNE	
B. thüringiensis			14	J. R. NORRIS	11
B. anthracis	Davis	Non sporulating	1	E. MEYNELL	1
		Capsulogenic	3	Own collection	12
		Non capsulogenic	6	Own collection	
		Defective prophage carrier	6	Own collection	5
B. subtilis	Marburg Yale		1	Own collection	
	NRS		3	R. E. GORDON	
B. subtilis var. atterimus	NRS		5	R. E. GORDON	
B. subtilis var. niger	NRS		3	R. E. GORDON	
B. licheniformis	NRS		5	R. E. GORDON	
B. megaterium	MUT-C	Phage resistant, non sporulating	1	Own collection	13
		Phage sensitive, Sporulating	2	Own collection	
			13	Own collection	

As an indicator for detecting phospholipase A produced either by a growing colony or one containing a lysate, a highly sensitive *B. megaterium* strain MUT-C was used. Phage β was assayed with strain Davis of *B. anthracis.*

Phages. Phage materials α and β were isolated from *B. cereus* W, and propagated either on strain Davis or VR of *B. anthracis.*

Anti-phage serum. The VR broth culture of *B. anthracis* was inoculated with phage (M.o.i.: 1.5) and aerated by shaking at 37°C until lysis, followed by centrifugation. After addition of 0.5 M NaCl and 5% polyethylene glycol (mol wt 6000) the precipitate formed was subjected to fractional centrifugation. The purified phage preparation from the ultracentrifuge contained 2.4×10^{12} particles. Altogether 4 rabbits were immunized, first intravenously, then intramuscularly with Freund adjuvant. The inoculation was generally performed with 1.2×10^{12} infective phages, every 5 days, five or six times. Individual rabbits gave a maximum value of 10—15 K.

Phage material wxc, the preparation of which is described in the experimental part, was applied to obtain antiphage serum. Rabbits were injected 4 times with 2×10^{10} virions in Freund adjuvant.

Culture media. Besides using the previously described YP (yeast extract-peptone) containing baker's yeast extract and peptone (Reanal, Budapest), the following media were used for comparative examinations: BPY: Beef extract, 3 g; peptone, 5 g; yeast extract, 3 g; NaCl, 3.5 g; distilled water, 1000 ml; pH 7.2. The ingredients were Difco products. $BPYe_1$: BPY was supplemented with bivalent cations: Mn^{++} 1/5000 M; Mg^{++} 1/1000 M; Ca^{++} 1/1000 M; Fe^{+++} 1/50 000 M. $BPYe_2$: besides containing the above-mentioned electrolytes, the following salts were added at a concentration of 1 μg/ml; $CoSO_4 \cdot H_2O$; $3CdSO_4 \cdot 8H_2O$; $ZnCl_2$. The media were solidified by the addition of 1.5% agar.

Cultivation of the bacteria. The same method was used as described previously [6]. When the culture reached an OD of 0.2—0.3, mitomycin C (MC) was added. After a residual growth the aerated cultures were lysed to a greater extent. The lysates were filtered (membrane filter, Göttingen, No. 6) and assay of the phage was performed as described previously [6]. The top agar layer contained 0.7% agar.

Isolation of plasmid-free clones. W bacteria were inoculated into YP containing either acridine orange or ethidium bromide (5—10 μg/ml) and inoculated under vigorous shaking at 37°C until they had formed spores. Spore materials were streaked to form 50—70 colonies, 1—2 mm in diameter, on YP agar at room temperature overnight. These master plates were replicated with the aid of velveteen disk on an agar plate in which the soft agar layer was seeded with *B. megaterium* MUT-C. The replicas were irradiated by Hanau low-pressure germicide lamps (250 erg/mm²) before incubation.

The MUT-C indicator layer was prepared by adding to 1 ml of melted YP agar 1 ml of bacterial culture with an OD of 0.5 and was put onto 25 ml bottom agar.

The clones which do not produce bacteriocin *(cin⁻)* were successfully isolated in this way. Attempts to produce β prophage-free derivatives have failed. Several ways were tried using *cin⁻* bacteria in the presence of either acridine orange or ethidium bromide and 0.05 M Na acetate at 37°C or 43°C to form spores. The cultures also contained 10% of antiphage serum. We have also tried, but without success, to treat the spores with a large dose of ultraviolet irradiation. Unfortunately we failed to select these β prophage-free clones when the *B. anthracis* Davis indicator strain was used according to the above-described method. Colonies treated by different procedures were therefore inoculated into a few ml of YP broth, cultured and centrifuged, and the supernatant was investigated for the presence of phages. In this way we could examine not more than a few hundred isolates.

Results

Production of plasmid-free derivatives of B. cereus strain W. Segregants which had lost the ability to produce bacteriocin were isolated with ease. Fig. 1 shows that while the wild type of clones have a marked halo, the *cin⁻* segregants have no effect on the indicator bacteria.

Table II lists the frequency of *cin⁻* segregants under different experimental conditions. The colonies seeming to be *cin⁻* on the indicator plates were checked by reisolation. It was found that about 2/3 of these isolates were stable, *e.g.* bacteriocin production could not be induced by the addition of MC. The *cin⁻* strains selected were maintained in the form of spores.

We attempted to isolate β prophage-free segregant by the described method, but failed to obtain even a single one from about 600 isolates. It seems that the prophage is integrated in the chromosome in a stable form.

Induction of the wild type strain and the cin⁻ derivative under different conditions. Induction of wild type and *cin⁻* strains was investigated under different conditions. MC was added in concentrations of 0.1 μg/ml and 0.5 μg/ml to young cultures. Samples were taken at intervals, and the number of

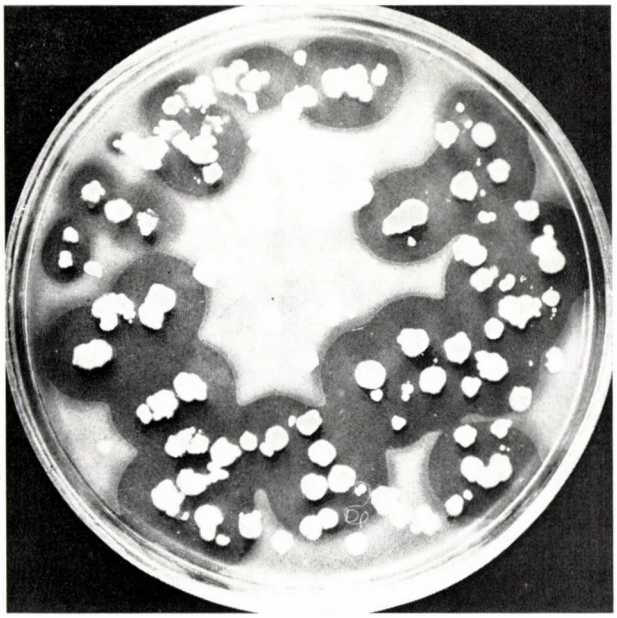

Fig. 1. Replicas on *B. megaterium* MUT-C of colonies formed from spore material obtained in the presence of acridine orange. The halo around the colonies indicates bacteriocin production. The colonies without halo are clones which have lost bacteriocin activity *(cin⁻)*. Original size

Table II

Frequency of cin⁻ segregant in B. cereus strain W sporulated under different conditions

Drug	No. of clones examined	*cin⁻* clones	
		No.	Per cent
O (control)	542	0	0
EB 5 μg/ml	502	132	26.4
EB 10 μg/ml	561	154	23.3
AO 10 μg/ml	1141	217	18.7

The experiment was performed on YP broth containing either ethidium bromide (EB) 5 μg/ml and 10 μg/ml or acridine orange (AO) 10 μg/ml. Cultivation was made until mass sporulation had occurred

plaque-forming units was determined on the Davis indicator plate. Fig. 2 illustrates the findings. Spontaneous induction of prophage β of the "wild type" strain W was insignificant. In the presence of 0.1 μg/ml MC, transitional lysis could be observed, accompanied by a significant phage production. In contrast, 0.5 μg/ml MC did not increase the phage release in spite of causing a marked lysis.

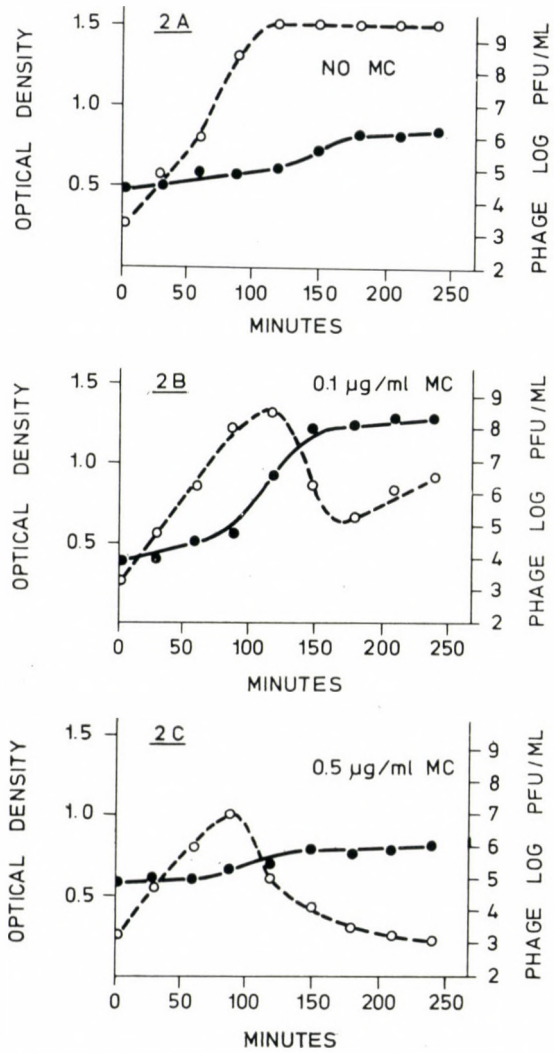

Fig. 2. Phage β production by *B. cereus* strain W under different conditions. (Indicator: strain Davis of *B. anthracis*). A. culture without mitomycin; B. 0.1 μg/ml; C. 0.5 μg/ml mitomycin. o = OD; • = p.f.u./ml

Phage-yield was insignificant in the absence of MC. However, in the presence of 0.1 μg/ml MC, the phage β yield was higher by 6 exponents. It was remarkable that in spite of the large phage-yield the optical density of the culture did not change. In the presence of 0.5 μg/ml MC, phage release was not more significant than in the cultures without MC, although growth was retarded (Fig. 3).

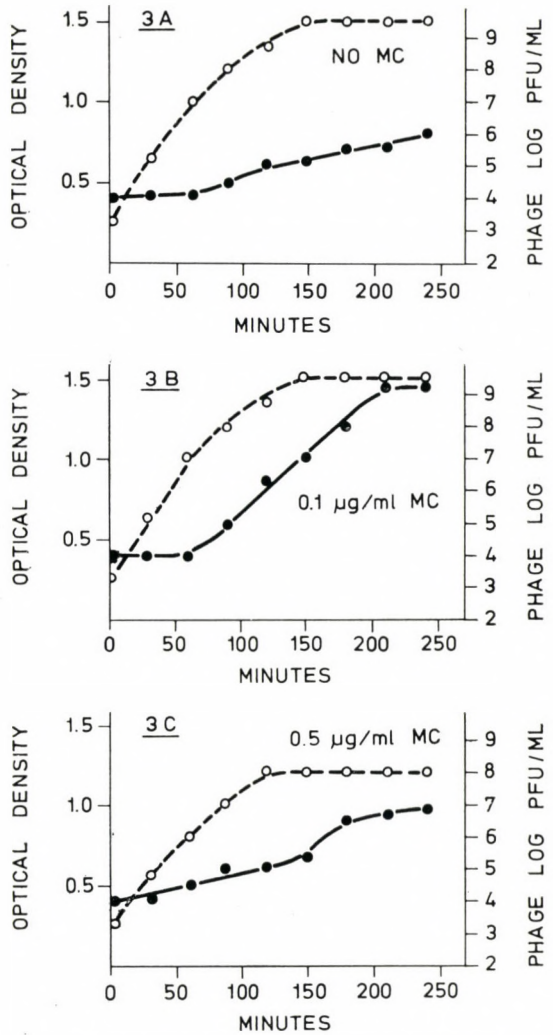

Fig. 3. Phage β production by strain *B. cereus* W *cin*$^-$ under different conditions. (Indicator: strain Davis of *B. anthracis*). A. culture without mitomycin; B. 0.1 μg/ml; C. 0.5 μg/ml mitomycin. o = OD; • = p.f.u./ml

The culture fluid of the wild type strain without MC displayed a bacteriocin effect at a titre of 1 : 100, similarly to that found when 0.1 μg/ml MC was applied. In contrast, when 0.5 μg/ml MC was present, even 1 : 3200 dilution of the lysate had an antibacterial effect.

These results suggest that at least two plasmids may occur in the wild type strain.

Isolation of phage wx. The lysates obtained from strain W under different conditions were implanted in *cin*$^-$ indicator cells, according to the usual

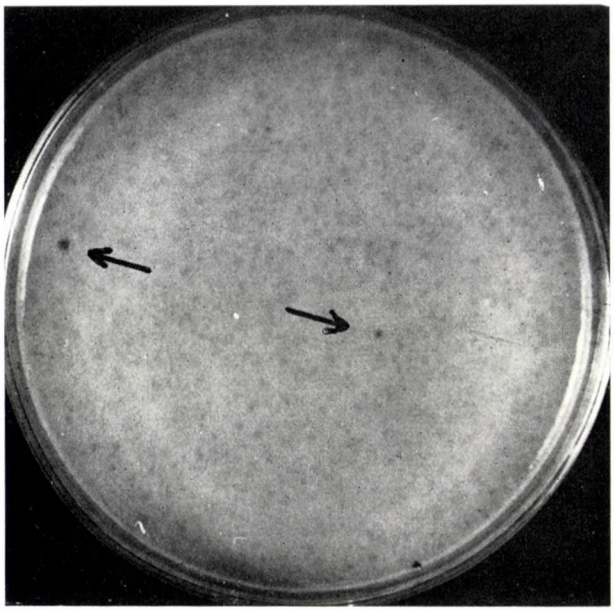

Fig. 4. Plaques of phage *wx* on *cin*$^-$ indicator strain. Besides numerous turbid plaques 2 clear mutants are seen (indicated by arrows). Original size

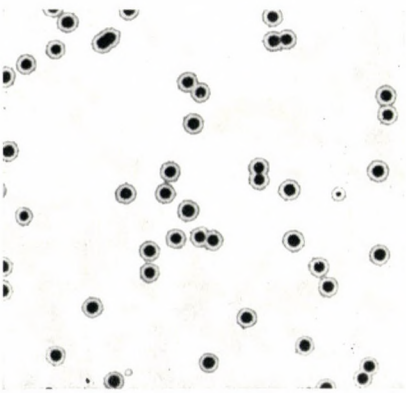

Fig. 5. Plaques of phage β on strain Davis of *B. anthracis* ($\times 1.5$)

method of phage-assay. The number of p.f.u. was determined simultaneously with Davis bacteria. Appearance and morphology of the plaques formed on the *cin*$^-$ indicator depended greatly on the medium applied. On YP medium, a large number of turbid plaques 1.0—1.5 mm in diameter were found. These plaques were different from those which had formed on Davis indicator plates (see Figs 4 and 5). When BPY medium was used, no plaque-formation was observed in the presence of *cin*$^-$ indicator. Certain cations seem to be necessary for phage propagation; this was clearly shown when $BPYe_1$ and $BPYe_2$ media

were used, when a similar plaque-formation resulted as on YP agar. When the BPY agar was supplemented with incinerated Reanal peptone, this provided the cations necessary for the formation of the characteristic plaques seen on YP agar.

The phages differed in their antigenic structure, too. Phage β could be neutralized by its homologous serum, in contrast to those phages which gave turbid plaques on *cin*$^-$ indicator plates.

This means that two phages were present in the lysate which differed in both antigenic structure and host specificity; one of these was the well-known phage β while the second phage was designated as *wx*.

It should be stressed that phage *wx* only formed plaques on *cin*$^-$ bacteria, which contains prophage β, and thus is immune to its vegetative phage.

The ratio of phages β and *wx* in individual lysates differed according to the concentration of mytomycin C used for induction (see Table III).

Table III

P.f.u./ml of phages wx and β in the lysates of B. cereus strain W

Phage	Indicator strain	Mitomycin C added to the culture		
		0.1 μg/ml	0.5 μg/ml	0
β	Davis	4×10^8	1.5×10^5	2×10^5
wx	*cin*$^-$	4×10^5	2.3×10^4	2×10^6

Phage *wx* was not effective either on the different wild-type isolates of *B. cereus* or the 11 *B. cereus* strains described previously [6]. Similarly, strains of *B. anthracis* were not attacked. The host range of *wx* will be described in connection with its clear mutant, since the lysate containing phage *wx* was associated with bacteriocin which often resulted in misjudgement.

Characteristics of the formation of clear plaques (*wxc*). Lysates of strain W induced with MC always contained a few clear plaques in addition to turbid one in *cin*$^-$ indicator plates. The morphology of this plaque is clear and at the edges the lysis is incomplete (Fig. 6). The individual plaques measure 3–7 mm in diameter. This typical morphology could be observed when both YP and $BPYe_2$ media were used. It was striking that on occasions even after 24 hr a pinhead-sized secondary colony developed in the middle of the plaque. A sporulated sample was taken from the secondary growth and phage contamination was inactivated by heat. When this was used as an indicator strain, it turned out to be completely resistant to phage *wx*. The host range of the *wxc* phage suspension was determined with numerous strains of the bacillus species. The phage suspension was diluted to contain 1.6×10^7 virions per 0.02 ml. The 97 strains listed in Table I were examined by inoculating the diluted suspension

of bacteria onto YP plate agar, after which drops of phage were applied. It was striking that none of the numerous strains of different *Bacillus* species were sensitive to phage *wxc*.

It must be pointed out that when the sensitivity of *B. anthracis* strainë was tested, the phage suspension was treated with Wα antiphage serum, and thus the phage Wβ virions occurring in the lysate had been neutralized.

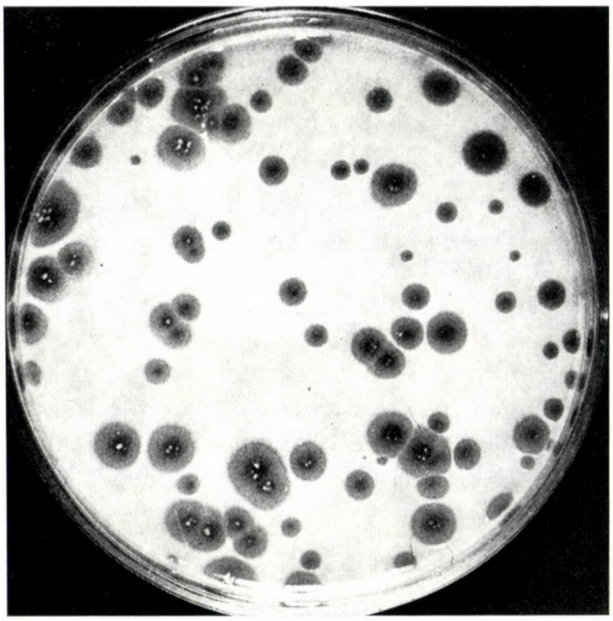

Fig. 6. Plaques of phage *wxc* on *B. cereus* W *cin*$^-$ bacteria. Original size

All *cin*$^-$ bacteria included prophage β, and this suggested that its presence might support propagation of phage *wx*. Strains of *B. anthracis* (VR and Sterne 18—74) were therefore lysogenized with phage β and infected with *wxc*; even in this case phage *wxc* failed to propagate.

Phage *wxc* resisted both RNA-ase and DNA-ase; when it was plated with *cin*$^-$ bacteria in a soft-agar layer containing either 5 μg/ml RNA-ase or 50 μg/ml DNA-ase, the number of plaques was identical with that of a control plate. The thermolability of the agent is also a proof in favour of the phage nature (see Fig. 7).

Phage *wx* can be considered a wild-type, an allelic mutant of *wxc*. The homologous serum of phage *wxc* neutralized the *wx* and *wxc* virions to the same extent.

Production of phage wx. A convenient method for obtaining wild-type virion *wx* was to induce a growing culture of strain W with 0.5 μg/ml MC. The

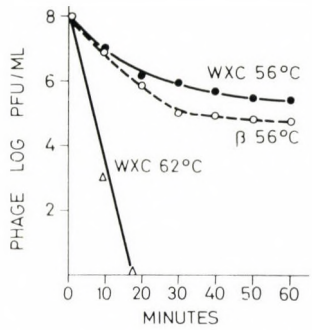

Fig. 7. Heat inactivation of phages β and *wxc*. The lysates were diluted 1 : 10 with 0.02M phosphate buffer pH 7.2

p.f.u. value of this lysate was about 10^6/ml, and could easily be increased by ultracentrifugation. The usual soft-agar method was applied to obtain a *wxc* phage suspension. About 10^3 phage particles were mixed with *cin*$^-$ bacteria into the soft-agar layer. The indicator bacteria were lysed overnight. The soft-agar layer was scraped off into saline containing 20% YP broth, under vigorous shaking and then centrifuged. In this way about 10^{10}/ml p.f.u. of *wxc* and 5×10^4 phage β were found in the lysate. Hence, the concentration of the *wxc* virions was about 5 orders higher than that of phage β.

Discussion

B. cereus strain W carries a prophage β [1–3], and when induced to lysis a bacteriocin is released which is identical with phospholipase A [6]. During these investigations a new factor has been observed, a prophage designated *wx*. Prophage *wx* can be induced by other conditions than those used for prophage β. There is no doubt that the two prophages represent two different genetic entities. The antigenic structure and some other characteristics of their virions are completely different. The most striking feature of phage *wx* is its extraordinary host-specificity. Numerous strains belonging to the genus *Bacillus* were not attacked, except for a derivative of *B. cereus* strain W, which does not produce bacteriocin. For this reason it seems reasonable to denote the prophage in question with the attribute "cryptic".

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DISTRIBUTION OF ^{131}I ANTIBODIES AND ^{125}I ANTIGENS IN ANAPHYLACTIC SHOCK OF THE GUINEA PIG

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(Received February 7, 1973)

Summary. Guinea pigs were sensitized with ^{131}I labelled isolated rabbit antibodies. The homologous antigen triggering the shock was labelled with ^{125}I. It was found that the quantity of antigen in the organs depended on the concentration of antigen in the blood of the animals. The ratio of rabbit anti-OA antibodies fixated to lungs and the bound antigen was 1 : 3—1 : 4 in cases of death due to anaphylactic shock. If the amount of bound antigen decreased, the shock reaction also decreased. On the other hand, on increasing quantity of antigen bound to antibodies even to 30-fold, the reaction did not change.

Double labelling of antibody and antigen has been applied for the examination of the special combination site of antibody [1] and for differentiating the binding of tissue antigen and specific antigen from the binding of non-specific IgG [2].

In the present experiments we have applied two kinds of label and a specific rabbit anti-ovalbumin-IgG for comparing the distribution of antibodies and antigens in the guinea pig's anaphylactic shock.

Materials and methods

Antigen. Four times recrystallized and gel-filtrated ovalbumin (OA).

Antibody. Rabbit IgG (specific IgG) isolated by immune-specific precipitation from rabbit serum hyperimmunized by OA in an incomplete Freund adjuvant [3].

Nonspecific IgG. Gel-filtrated IgG prepared by the ammonium sulphate method from the serum of untreated rabbits.

Labelling of proteins with radio-active iodine. The rabbit anti-OA IgG and the nonspecific rabbit IgG were labelled with ^{131}I, and the OA was labelled with ^{125}I, by the chloramine T method [4]. The guinea pigs were treated as follows. Sixteen guinea pigs were given intracardially 480 μg N/kg body weight of the isolated antibody, 16 guinea pigs were given the same amount of non-specific rabbit IgG. After 48 hr, of each group 8 animals were given 3 mg, and the other 8 animals 0.3 mg labelled OA intrajugularly. Before death due to shock, i.e. 2—5 minutes after the OA injection the animals were bled to death. The activity of 0.5 ml blood and 0.5—1 g tissue sample was measured. Oxyhaemoglobin (OHb) in the organ samples was determined according to the method described by TÓTH and JÓKAY [5].

Measuring of ^{131}I and ^{125}I. Eighty per cent of the gamma rays from ^{131}I has an energy of 0.36 MeV, while the energy of gamma and X-rays from ^{125}I is 0.035 MeV. Hence they can easily be determined next to each other. At the same time 20% of the radiation from ^{131}I covers the radiation of ^{125}I. Therefore we have measured standards of ^{131}I and ^{125}I of which the counts were exactly known, separately and jointly. From these measurements we calculated how many of the counts originated from ^{131}I and from ^{125}I in our own system. This was 19.4%. The activity of the samples was measured in 1 ml volume corresponding to the standard, on a one-channel scintillator in such a way that every sample was placed twice into the measuring system for estimating the higher and the lower energy. The 19.4% of the ^{131}I activity was subtracted from the number of counts due to ^{125}I activity. The activities were reduced to N by means of the calibration curve.

Results

In Fig. 1 two neighbouring columns display the distribution of the specific anti-OA rabbit antibodies and of the non-specific rabbit IgG in the tissues and blood of the guinea pigs. The immunoglobulin quantity corresponding to the blood content of the organs was subtracted from the antibody content of the tissues.

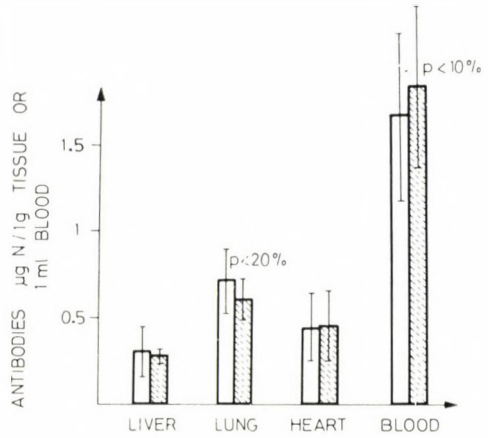

Fig. 1. Distribution of rabbit IgG in the tissues of guinea pigs 48 hr after IgG administration. □ = 480 μg N rabbit anti-OA IgG/kg ▨ = 480 μg N non-specific rabbit IgG/kg

Fig. 1 shows that the quantity of specific and non-specific immunoglobulin was nearly equal in blood and tissues.

Fig. 2 indicates the distribution of immunoglobulins and antigens in the identical organs and in the blood of a guinea pig treated with specific IgG and a guinea pig treated with non-specific IgG after an injection of 3 mg OA. Although IgG is bound to organs to a larger extent than is OA, in the organs of the control animals the quantity of OA was higher than that of IgG. This may have been due to the fact that the OA content of blood was 15-fold of that of IgG.

Fig. 3 displays the distribution of antibodies and antigen in guinea pigs. These guinea pigs were treated with immunoglobulins in a similar way as the previous ones except that they were given 0.3 mg of OA.

The antigen content of the control organs decreased to nearly zero, the liver of the animal treated with specific IgG hardly contained any OA. The antibody-antigen molecule ratio in the lungs was 1 : 4. The animals presented in Figs 2 and 3 had been sensitized by antibodies and they responded to OA with shock, therefore they had to be bled to death just before death had set in.

Finally, Fig. 4 shows the quantity of OA in the lungs and liver in correlation to the antigen circulating in the blood of guinea pigs sensitized by specific

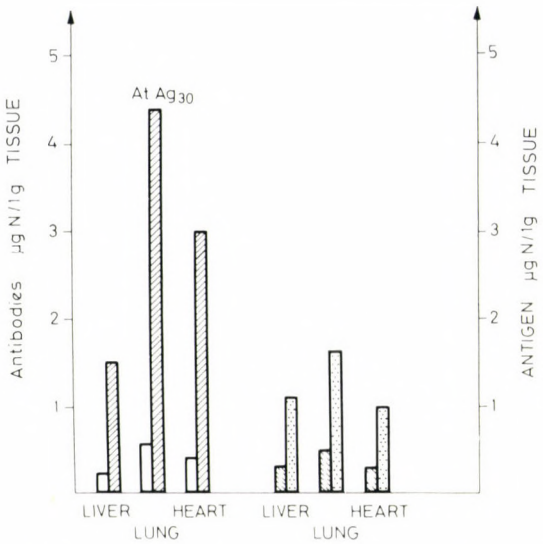

Fig. 2. Distribution of rabbit IgG and of ovalbumin in organs of guinea pigs 48 hours after IgG administration. □ = 480 µg N rabbit anti-OA IgG/kg; ▨ = 480 µg N non-specific rabbit IgG/kg; ▨ = 3 mg OA in guinea pig treated with specific antibodies; ▨ = 3 mg OA in guinea pig treated with non-specific IgG. The antibody content in the blood of the guinea pig treated with antibodies was 1.96 µg N/ml, the OA content was 27.50 µg N/ml. The IgG content of blood of the guinea pig treated with non-specific IgG was 2.05 µg N/ml, the OA content, 30 µg N/ml

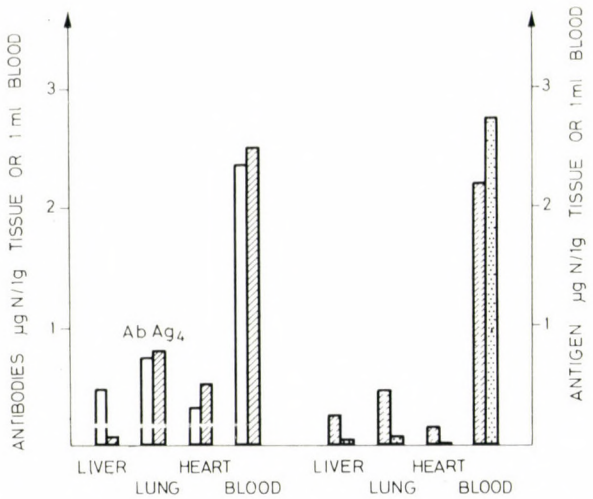

Fig. 3. Distribution of IgG and of ovalbumin in tissues of guinea pigs 48 hours after IgG administration. □ = 480 µg N anti-OA rabbit antibody/kg; ▨ = 480 µg N non-specific rabbit IgG/kg; ▨ = 0.3 mg OA in guinea pig treated with OA; ▨ 0.3 mg OA in guinea pig treated with non-specific IgG

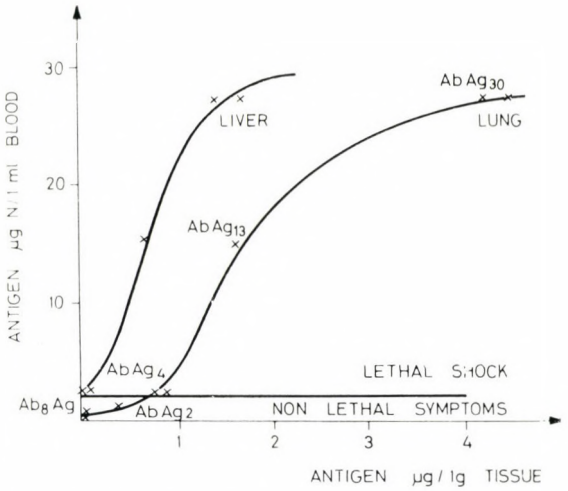

Fig. 4. Antigen content in liver and lungs in correlation to the quantity of antigen in blood The antibody-antigen proportion in the lungs is also shown. Points indicate means of paralle measurements

antibodies and treated with different quantities of OA. The ratio of antibody-antigen measured in the lungs is also shown. The least severe shock symptoms were observed in the animal which had in its lung antibody and antigen in a proportion of 1 : 2.

Discussion

Although part of the antibody detected in tissue can be removed by repeated washing, in the present experiments we did not try to determine the absolute quantity of the strongly fixed antibodies. Our aim was to compare the binding of specific and non-specific antibodies and to determine the proportion of antibody and the fixed antigen in the organs, under identical experimental conditions.

The ratio of fixed antibody and antigen binding is significant from the point of view of the guinea pig's reaction in systemic anaphylaxis although a large antigen excess did not inhibit its development. According to CÎRSTEA [6], in systemic anaphylaxis the reaction depends only slightly on the antigen dose. Our own findings in systemic anaphylaxis indicated that the biological effect was due to a given quantity of bound antibodies reacting with a definite quantity of antigens. When performing a Schultz-Dale test the changes taking place on the membrane of the muscle fibrils are proportional to the antigen-binding capacity of the sensitizing (bound) antibodies. OPAKO [7] studied the dependence on the antigen-dose of the contractility of the sensitized guinea pig ileum, and found that a too large amount of antigen inhibited the reaction.

SHELDON and HICKS [8] observed that a minimum dose of antigen increased the reaction; if the antigen dose was increased, the response reached a maximum and after further increasing the amount of antigen, the reaction tended to decrease.

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AUSTRALIA ANTIGEN SURVEY IN HUNGARY

IV. ANTIBODIES TO TWEEN-80-TREATED AUSTRALIA ANTIGEN AND TO ALLOGENEIC LIVER FRACTIONS IN PATIENTS WITH HEPATITIS

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(Received March 10, 1973)

Summary. Dane particles were partially purified by differential centrifugation from pooled sera of healthy HBAg carriers. The suspensions thus obtained were treated with Tween 80 and used as antigen (THBAg) in the complement-fixation test. Specific complement fixing antibody (THBAb) was detected at a considerable frequency in serum samples from HBAg-positive patients with acute hepatitis and those with chronic hepatic disease, *viz.*, in 19% of 279 cases of acute hepatitis from each of which a single blood sample was tested; in 29% of 136 cases of acute hepatitis from which serial samples were tested; in 10% of 371 convalescents tested after discharge from hospital; in 53% of 74 patients with chronic persistent or chronic aggressive hepatitis or hepatic cirrhosis. The course of the acute illness in THBAb-positive cases did not differ from that in negative cases. Patients who already had detectable antibodies at the onset of clinical symptoms tended to recover earlier. Demonstration and follow-up of THBAb in patients with acute hepatitis and in those with chronic hepatic disease may serve as an additional tool in the understanding of the pathomechanism and, perhaps, in the differential diagnosis of hepatic diseases.

Almeida *et al.* [1], using immuno-electron microscopy, demonstrated in serum samples from hepatitis patients a long-persisting antibody different in specificity from the anti-Australia antibody (HBAb). They supposed that the new antibody, which reacts with the inner component of Dane particles, may play some role in recovery.

In a previous study [2] we treated semi-purified Dane particles with Tween 80 to reveal the internal component. The preparation thus obtained was examined by electron microscopy and by the complement fixation (CF) test, using HBAb-positive haemophiliac serum as antibody.

We have taken into consideration that immuno-electron microscopy cannot be used for mass investigations. Therefore, we have adopted the micro-CF test for the demonstration in human sera of antibodies reacting with the Tween-80-treated (THBAg) preparation. In the present work single and serial serum samples from patients with hepatitis and those with chronic hepatic disease were tested for antibodies (THBAb) reacting with THBAg and for allogeneic antiliver antibodies.

Materials and methods

Antigens. Serum from a patient with Down's syndrome was used as HBAg. THBAg was prepared from partially purified Dane particles by treatment with Tween 80 as described elsewhere [2]. Although the Dane particle preparation as tested by the CF test had a lower

HBAg activity than the unselected HBAg, we had to reckon with both HBAg and THBAg activities in our THBAg preparation. The antigens and antisera were stored at —60°C and a new ampoule was opened on each day when tests were performed.

Complement fixation test. TAKÁTSY's micro-test was employed as in our previous studies [2, 3]. Sera showing HBAb and/or anticomplementary (AC) activity were not considered positive for THBAb unless their THBAb titre exceeded both of the former titres by at least one dilution step.

Control preparations from HBAg-negative sera. Attempts were made to prepare a Tween-80-treated preparation from two pools of HBAg-negative sera. However, the first preparation proved to be AC, the second one slightly positive for HBAg. To avoid further failures, individual sera were centrifuged similarly as the pools [2] and the non-AC HBAg-negative sediments were pooled. The control antigen gave no CF reaction with either the HBAb-positive haemophiliac serum or with sera from 15 patients with chronic persistent hepatitis each having high THBAb titres. The control antigen was not examined by electron microscopy.

Assay for antibodies to hepatic subcellular fractions. The passive haemagglutination micromethod was used as described by SURJÁN and NYERGES [4]. The antigens were prepared from the liver of a 21-year-old victim of an accident, using the method of SZÉCSEY *et al.* [5]. The nuclear fraction (NF) and the mitochondrial fraction (MiF) were prepared by centrifugation for 1 hr at 2000 *g* and 20 000 *g*, respectively. The supernatant after a 1-hr centrifugation at 100,000 *g* served as a soluble protein fraction (SF). From the sediments, 2% suspensions were prepared; protein concentration in the SF was also 2%.

Patients. Group A (279 cases) and Group B (136 cases) consisted of HBAg positive cases of acute hepatitis. In group A a single blood sample was examined from each of the patients. The interval between the onset of illness and the day of blood sampling was not registered. From each of the patients in group B one sample was taken within 5 days after admission and subsequently several samples at 7 to 14-day intervals until discharge.

Of the acute cases, 32 THBAb-positive and 66 THBAb-negative patients were tested for allogeneic anti-liver antibodies.

Group C consisted of 371 convalescents. These were tested 2 to 4 times in the period from the 30th day to the 200th day after discharge. All these had been HBAg-positive during the acute phase of their illness. In this group, 73 patients including 37 THBAb-negative ones were tested for allogeneic anti-liver antibodies.

Group D included 10 patients of chronic aggressive, 68 patients of chronic persistent hepatitis and 49 patients with liver cirrhosis of different origin. All these were tested once for HBAg, HBAb, AC activity THBAb and antibodies to allogeneic anti-liver antibodies. Only the cases positive for HBAg or HBAb have been analyzed in detail.

In 10 cases of aggressive chronic hepatitis, 45 cases of persistent hepatitis and 32 cases of cirrhosis the diagnosis has been confirmed by liver biopsy. In the rest the clinical picture and the laboratory tests seemed to be sufficient for correct diagnosis.

Results

Group A. Of the single serum samples obtained from the 279 patients only 52 (19%) contained detectable amounts of THBAb (Table I). The percent-

Table I

THBAb in single serum samples from 279 HBAg-positive patients with acute hepatitis

Group	THBAb			Anti-complementary sera		No. of cases tested
	negative	positive				
	No.	No.	per cent	No.	per cent	
Children 2—15 years	78	21	18	15	13	114
Men 16—80 years	62	15	17	12	13	89
Women 16—80 years	42	16	21	18	24	76
Total	182	52	19	45	16	279

age of positive cases was approximately the same for males, females and children. Forty-five samples were AC; in these samples the THBAb test could not be evaluated.

Table II

THBAb in serial serum samples from 136 HBAg-positive patients with acute hepatitis

Group	THBAb			Anti-complementary sera		No. of cases followed up
	negative	positive				
	No.	No.	per cent	No.	per cent	
Children 2—15 years	19	12	30	9	23	40
Men 16—80 years	26	15	31	7	15	48
Women 16—80 years	30	12	25	6	13	48
Total	75	39	29	22	16	136

Group B. In this group 39 patients (29%) had THBAb in at least one blood sample (Table II). In further 22 cases (16%) the presence of THBAb could not be excluded because of AC activity and/or the presence of HBAb.

In Table III the 39 THBAb-positive cases are analyzed from three points of view: (i) the day of the first positive finding as related to the day of admission; (ii) the length of the period during which THBAb was demonstrable; (iii) the possible relationship between the disappearance of THBAb and recovery.

On this basis the 39 THBAb-positive cases were divided into three groups. Those in group I had detectable antibody only in the first blood sample taken in the hospital.

Cases in which THBAb was demonstrable in more than one sample were classified into group II; those in which the first sample was negative and one of the subsequent samples was positive were classified into group III. In some of the cases in groups II and III, the time of disappearance of THBAb could not be established because the patient had left the hospital.

Of the 40 children 12 (30%), of the 48 men 15 (31%) and of the 48 women 12 (25%) had THBAb in at least one serum sample.

Group I included 6 children, 4 men and 2 women. The average period of hospital care was 23 days (mean, 23 days), indicating that the majority of these patients recovered in a relatively short time.

Group II included 4 children, 4 men and 6 women. One man and one woman died, both of hepatic coma; for the others the average period of hospitalization was 34 days (mean, 28 days). The man who died had a THBAb-negative sample two days before death; the woman had alternating positive and negative results.

Table III

First demonstration and persistence of THBAb in serum samples from HBAg-positive patients with acute hepatitis

Group	Patient No.	First positive sample		Day of first negative sample	Day of discharge	Remarks
		day	reciprocal titre			
A *Children*						
	1	4	16	10	24	
	3	5	8	15	16	
I	7	4	2	12	27	
	9	3	16	10	25	
	11	3	8	13	27	
	12	3	16	15	29	
	2	19	2	—	28	
		25	8	—	28	
II	5	4	12			
		19	6	26	27	
	6	3	6	—		
		11	8	23	25	
	4	12	12	27		Treatment continued
III	8	19	4	—	22	
	10	44	3	—	47	
B *Adult males*						
	3	3	4	15	19	
I	6	4	2	20	22	
	7	4	16	15	17	
	8	4	2	19	21	
	2	10	8	—		
		29	16	—	33	
II	5	5	6	—		
		11	3	—	12	
	9	4	16	—		
		18	2	—	21	
	14	10	8	—		Alternating positive
		17	6	25		and negative samples
		31	4	38		Died on 40th day
	1	15	6	24	26	
	4	9	16	12	14	
III	10	22	8	29	30	
	11	12	3	19	25	
	12	69	3	75	137	Treatment continued
	13	25	2	—		Died on 28th day
	15	48	3	—	50	

Group	Patient No.	First positive sample		Day of first negative sample	Day of discharge	Remarks
		day	reciprocal titre			
C *Adult females*						
I	1	4	6	26	30	
	3	3	2	14	18	
II	4	3	2	—		
		59	4	—	62	
	5	4	12	—		
		13	8	22	24	
	10	20	3	—		
		27	2	34	35	
	11	24	2	—		
		31	3	38	52	
	12	5	3	—		Alternating positive and negative samples
		12	3	—		
		33	2	—		Died on 36th day
	8	37	3	—		
		45	8	—	50	
III	2	43	12	—	48	
	6	15	8	—	20	
	7	14	2	—	20	
	9	31	2	45	52	

Group III included 2 children, 7 men and 4 women. One of the male patients died of hepatic coma a few days after the first demonstration of THBAb. The average period of hospital care was 40 days (mean, 28 days).

None of the 97 patients with no THBAb positivity in their serum samples died, and the average period of hospital care for this group was 34 days. The serum samples from 22 of these patients were AC which might have masked THBAb.

For allogeneic anti-liver antibodies one serum sample from each of 11 children, 14 men and 7 women, all THBAb-positive patients, was tested. One child (No. 2 in group II) had anti-nuclear antibody in the sample taken on the 19th day. The THBAb titre of the same sample was 1 : 2. One man (patient No. 9 in group II) had antibodies to the SF in the sample taken on the 4th day (THBAb titre, 1 : 16). All the others were negative.

Among the 66 THBAb-negative patients tested for allogeneic antigens, 3 had antibodies to both MiF and NF. All the other tests were negative.

Patients followed up after discharge. Three hundred and seventy-one patients were examined for THBAb 1 to 2 months after having left the hospital (Table IV). From part of them serial samples were available. THBAb was demonstrable in the serum of 37, and 29 serum samples were AC. Both THBAb and AC activity was the most frequent in adult male patients.

Fifteen male patients were tested both in the acute phase and after discharge. Of these 9 were consistently negative for THBAb, 5 were negative in

Table IV

Patients followed up after discharge

Group	THBAb			Anti-complementary sera		No. of cases tested
	negative	positive				
	No.	No.	per cent	No.	per cent	
Children 2—15 years	60	2	3	2	3	64
Men 16—80 years	139	28	15	19	10	186
Women 16—80 years	106	7	6	8	7	121
Total	305	37	10	29	8	371

the acute phase, but THBAb was demonstrable in their serum on the 96th, 140th, 62nd, 60th and 116th day after onset, respectively. In one case THBAb was present from the 10th to the 84th day, *i.e.* over a period of at least 75 days. From 32 male patients only a convalescence sample was available. Of these four proved to be positive. In one case THBAb was present over at least 100 days.

Twelve female patients were tested both in the acute phase and after discharge. Of these nine were consistently negative, two had THBAb only in the hospital, whereas one had the first positive sample on the 86th day after onset.

There was no appreciable relationship between the length of antibody persistence and the course of the illness.

Fifteen children, 38 men and 20 women were examined for allogeneic anti-liver antibodies after discharge. All the 73 patients including 37 THBAb-positive ones proved to be negative.

Chronic hepatic diseases. Of 68 cases of chronic persistent hepatitis, 33 (49%) proved to be HBAg-positive. Five, including one who also had HBAg, were positive for HBAb. Thus, 37 patients (54%) may be considered positive for the HB system. Of the 37 patients 17 (46%) had THBAb in their serum and 25 (67% !) had AC serum. Fourteen, 12 and 7 patients had allogeneic antibodies to the liver cell fractions NF, MiF, and SF, respectively.

Ten patients suffering from chronic aggressive hepatitis have been examined. All proved to be HBAg-positive, two HBAb-positive and five had THBAb. All had allogeneic antibodies to the SF and eight to NF as well.

Of the 49 patients with hepatic cirrhosis 22 (45%) proved to be HBAg-positive, and five HBAb-positive; of these 27 patients 17 proved to be positive for THBAb. The frequency of allogeneic antibodies was similar to that for patients with chronic persistent hepatitis.

In HBAg-positive (or HBAb-positive) chronic hepatic disease, THBAb positivity was considerably more frequent than in the patients with acute

hepatitis followed up after discharge. Among the 74 chronic cases positive for HBAg or HBAb 39 (53%) had also THBAb in serum.

Mean age of the patients suffering from chronic aggressive hepatitis, chronic persistent hepatitis and hepatic cirrhosis was 41, 51 and 58 years, respectively.

Table V

Laboratory results for chronic cases

Group	No. of cases tested	HBAg	HBAb	AC	THBAb	Sero-negative	Positive for allogeneic ab			AC + allo-geneic ab	THBAb + alloge-neic ab	AC+ THBAb	Mean age
		positive					SF	MiF	NF	positive			
						a) Chronic persistent hepatitis							
Females	46	21	3	17	9	14	5	7	9	7	4	3	49
Males	22	12	2	8	8	8	2	5	5	3	4	5	55
Total	68	33	5	25	17	22	7	12	14	10	8	8	51
						b) Chronic aggressive hepatitis							
Females	6	6	1	5	2	0	6	5	5	5	2	1	39
Males	4	4	1	3	3	0	4	4	3	3	3	3	42
Total	10	10	2	8	5	0	10	9	8	8	5	4	41
						c) Liver cirrhosis							
Females	14	6	2	8	5	5	3	4	5	4	2	3	63
Males	35	16	3	19	12	6	3	4	4	4	3	8	56
Total	49	22	5	27	17	11	6	8	9	8	5	11	58

As shown in Table V, the frequency of allogeneic antibodies and of multiple positivity was much higher in chronic aggressive hepatitis than in either chronic persistent hepatitis or hepatic cirrhosis.

Discussion

The CF test for the demonstration of THBAb is less sensitive than immuno-electron microscopy. Besides, 16% of the patients in the acute phase of hepatitis and 8% of the convalescents had AC activity and/or HBAb in their serum and these might have masked THBAb. Thus the number of THBAb-positive patients must have been higher than that registered in the present study. In spite of this, it seems to be justified to suppose that patients who had demonstrable THBAb in their serum early during the acute phase of their ill-

ness, perhaps already in the prodromic period (group I in Table III), tended to recover earlier than those developing THBAb later or not at all. Supposedly, the appearance of THBAb at an adequate time may be favourable, whereas its inadequate appearance and/or relatively long persistence may be disadvantageous, for the course of the disease. In accordance with this, in all the three fatal cases THBAb was demonstrated in one or more serum samples. This seems to be a mechanism resembling that suggested for HBAb in the pathomechanism of hepatitis B.

In some cases THBAb was demonstrable for long periods of time. The present data failed to provide evidence of a relationship between THBAb persistence and the course of the disease. The fact that allogeneic antibodies occurred in THBAb-positive and THBAb-negative patients at approximately the same frequency speaks against such a relationship. On the other hand, the high incidence of THBAb in the chronic cases seems to be in accordance with the view that the persistence of THBAb-positivity may be an unfavourable sign.

In the material of BOND and HALL [6] the ratio of 20-nm particles to 45-nm particles was estimated at 1000 : 1. Our preparation technique was favourable to the 45-nm particles as shown by the electron micrographs in our previous study [2]. It was also shown that the HBAg CF activity became weaker during preparation. In this reduction, besides the lower antigenicity of the larger particles, the loss of protective colloids might have played a role [6, 7].

The fact that THBAb was found in the serum of one-third of the acute HBAg-positive patients and in 50% of the chronic cases suggests that the carrier of the THBAg, *i.e.*, the large particle might play a role in the pathomechanism of the HBAg-positive cases of hepatitis.

Demonstration and follow-up of THBAb in the serum of patients with acute hepatitis and of those with chronic hepatic disease might serve as an additional tool in understanding the pathomechanism and, perhaps, in the differential diagnosis of these diseases.

Addendum. J. O. NIELSEN *et al.* [New Engl. J. Med. **228,** 484 (1973)] have recently shown that Dane particles occurred in the serum of chronic hepatitis patients more frequently than in that from mild acute cases or healthy donors. Supposing that antibodies against intracellular antigens are not produced unless these antigens are released, this morphological observation is in accordance with our serological results in that antibodies to Dane particles have been found more frequently in chronic than in acute cases.

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WHOLE BODY COUNTING OF ^{32}P-ENDOTOXIN ELIMINATION IN ENDOTOXIN-TOLERANT AND CONTROL MICE

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Summary. Tolerance was induced in mice by endotoxin pretreatment, and the elimination of ^{32}P-endotoxin has been studied with whole body counting. The endotoxin disappeared slower from animals displaying increased tolerance and higher survival rate.

Studies on the elimination of endotoxin have been dealing so far with the disappearance of different isotope-labelled endotoxins from blood and with their distribution in the organism [1–3].

In the present studies the correlation between the effective half life (T_{eff}) of ^{32}P labelled endotoxin and the endotoxin tolerance of mice has been investigated.

Labelling with ^{32}P of *Escherichia coli* endotoxin was done by biosynthesis; a method which ensured the most stable labelling [4].

The kinetics of elimination was studied *in vivo* by means of the whole-body-counting technique, on the basis of strong beta particles induced bremsstrahlung developing in the tissues of mice [5–7].

Materials and methods

Animals. Female mice of our own strain, weighing 20 to 25 g were used. The animals were given mouse chow and water *ad libitum*.

Preparation of endotoxin. For pretreatment, endotoxin was prepared with the technique of Boivin and Mesrobeanu [8] from *Escherichia coli* O124. The LD_{50} of the endotoxin was 0.5 ml. ^{32}P labelling was carried out by the biosynthesis technique of Szabó *et al.* [4] as modified by Dzvonyár *et al.* [3]. According to this modification, the $Na_2H^{32}PO_4$ which provided the labelling as well as ensured the pH, was added to the shake culture. The count rate of 1 ml dried endotoxin was 5×10^5 cpm as determined in the GM tube. Taking into consideration the degree of efficiency of the measuring assembly, specific activity was 0.535 μCi/0.1 ml. The labelled endotoxin contained LD_{70}/24 hr in 0.2 ml.

Measurement of ^{32}P endotoxin disappearance. The elimination of labelled endotoxin was estimated *in vivo* by whole body counting. Whole body counting represents a technique by which the radioactive radiations emerging from the live organism are measured with geometry factors and efficiency is determined in a 4π or smaller angle. In the experiments, the bremsstrahlung of high energy ^{32}P beta particles was detected with an efficiency of $\eta = 4.2\times10^{-2}$. Using a lead collimator designed at our Institute [7], a one-crystal total body counter with reduced background was constructed (Fig. 1) which, when appropriate detectors were applied, was suitable for the measurement of middle and low energy γ radiation as well as of bremsstrahlung. The smallest demonstrable activity (A_m) of bremsstrahlung induced by ^{32}P beta particles was $A_m = 3.5\times10^{-2}$ μCi in the measuring assembly, as calculated by the equation,

$$A_m = \frac{k}{s}\sqrt{\frac{B}{t}}$$

where k represents the safety factor (k = 3 in our case); s = cpm, corresponding to 1 μCi; t = measuring time in minutes; B = cpm of the background.

We succeeded in measuring *in vivo* the disappearance kinetics of high-energy pure beta-active nuclides and that of proteins and polysaccharides labelled with them, by producing an endotoxin of high specific activity and by increasing the level of detection.

Treatment. Elimination of ^{32}P labelled endotoxin has been studied in three groups of mice, after physiological saline and unlabelled endotoxin treatment. Each group consisted of 26 animals. Mice of the first group were given 0.1 ml of physiological saline intraperitoneally. The second group was treated intraperitoneally with 0.1 ml of tenfold diluted endotoxin, containing $LD_{50}/24$ hr in 0.5 ml, thus with the fiftieth part of $LD_{50}/24$ hr. Animals of the third group received by the same route 0.1 ml undiluted endotoxin, *i.e.*, one fifth $LD_{50}/24$ hr. The treatment was given every other day, on 6 occasions. The degree of endotoxin dilution is presented in Table I. Two days after the last treatment each group was injected intraperitoneally with 0.2 ml of ^{32}P-endotoxin. Activity was measured in the whole body counter 2 hr after treatment on five consecutive days. The elimination curve was plotted from the data of animals surviving five days. Although only 4 of the controls and 10 and 16 of the tolerant animals lived on the last day, the curve could still be plotted, as the highest and lowest values for the different groups did not overlap.

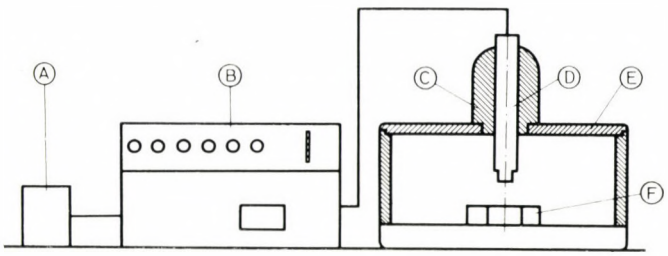

Fig. 1. Diagram of measuring assembly: A = Time-switch chronometer (accuracy 10^{-4} s/hr); B = Decadal counter (Siemens system); C = Lead shield for detector; D = Roentgen-crystal detector (Type 3 S 114055); E = Lead wall of whole body counter collimator, thickness: 35 mm; F = Container made of 0.2 mm thick plexiglass ensuring circular track for the animals, providing thus standard measuring geometry

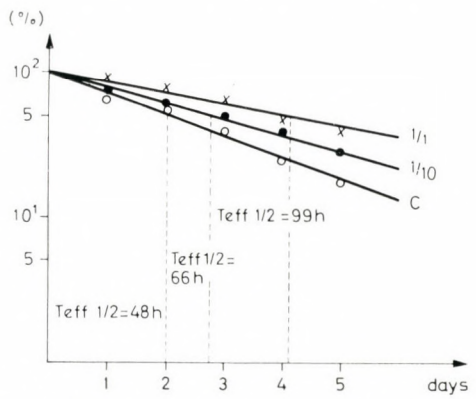

Fig. 2. Effective half-period of ^{32}P-endotoxin in mice. Determination of the effective half-period of ^{32}P-endotoxin, in mice made tolerant to different degrees and in controls. Semi-logarithmic presentation of pulses counted 2 hr after ^{32}P endotoxin treatment. C = control group; 1/10 = group pretreated with 0.1 ml of tenfold diluted endotoxin containing $LD_{50}/24$ hr in 0.5 ml; 1/1 = group pretreated with 0.1 ml of undiluted endotoxin cotaining $LD_{50}/24$ hr in 0.5 ml

Results

The effective half-life of ^{32}P labelled endotoxin was the shortest in the control group, longer in animals pretreated with diluted endotoxin and longest in the animals treated with undiluted endotoxin (Fig. 2).

The 0.2 ml quantity of ^{32}P-endotoxin equalled LD_{70}/24 hr in the animals pretreated with physiological saline. Endotoxin pretreatment was found to reduce the lethality rate. Diluted endotoxin induced a moderate, and undiluted endotoxin pretreatment a pronounced, tolerance of the labelled endotoxin. Results are summarized in Table I.

Table I

The effect of pretreatment with diluted and undiluted endotoxin

Treatment	No. of animals	No. of survivors	$T_{eff(hr)}$
Physiological saline control	26	4	48
1 : 10 diluted endotoxin	26	10	66
Undiluted endotoxin	26	16	99

Discussion

The use of labelled endotoxin of high specific activity and of the most suitable detector allowed reliably to follow the elimination of ^{32}P-endotoxin. Its quantity was determined by the specific activity and the efficiency of the measuring assembly. The effective half-life of ^{32}P-endotoxin was prolonged by inactive endotoxin pretreatment which decreased the lethality rate and increased the endotoxin tolerance of the animals.

These results *in vivo* have supported the findings obtained in isolated organs [1], indicating that the tissular fixation of phagocyted endotoxin particles increased in consequence of an activation of the reticulo-endothelial system by endotoxin pretreatment [2, 9–11].

A close connection was found between the increase of tolerance on endotoxin treatment and of the effective half-life of endotoxin. Owing to the increased RES activity the endotoxin disappeared slower from the organism displaying an increased tolerance.

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ANTIVIRAL EFFECT OF 1-AMINOADAMANTANE DERIVATIVES IN VITRO

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Summary. Inhibition by 1-aminoadamantane derivatives of the reproduction of the influenza A(H2N2)Singapore(1)57 and parainfluenza-1 (Sendai) viruses in chicken chorioallantoic membrane has been investigated. N-methylene aryl derivatives proved to be less effective than 1-aminoadamantane hydrochloride. The antiviral activity of the former compounds depends on the substituting aryl radical. The thioglycollate of 1-aminoadamantane was found to be as active as, and its mercaptopropionate slightly more active than, the hydrochloride.

1-Aminoadamantane (amantadine) was first synthesized in 1941 [1]. Its antiviral effect was described in 1964 [2]. The compound was found selectively to inhibit *in vitro* and *in ovo* some members of the Myxovirus group, to the highest degree some strains of influenza A virus, and to exert a protective effect *in vivo* in mice infected with influenza virus.

A protective effect was also demonstrated in field trials carried out during epidemics caused by influenza A/H2N2 virus strains. In subjects treated with amantadine the clinical picture was milder, the course of the illness shorter and the frequency of complication lower than in the control groups [3–9]. The drug seems to be less effective against the strains containing H3 haemagglutinin (so-called Hong Kong virus strains).

Recently, numerous amantadine derivatives have been synthesized. Of these, rimantadine (alpha-methyl-1-adamantane-methylamine) proved to be more effective *in vitro* than amantadine [10] and it seemed to have a prophylactic and therapeutic effect in volunteers infected with influenza A/H2N2 virus [11, 12].

Materials and methods

Tissue culture. Chorioallantoic membrane (CAM) fragments from embryonated hen's eggs surviving in a medium containing phosphate-buffered saline, glucose and egg-white [13].

Viruses. The strains influenza A(H2N2)Singapore(1)57 and parainfluenza-1 (Sendai) were maintained in serial allantoic passages in chick embryos. The allantoic fluids containing virus were stored at −70°C.

Chemicals. Chemical structure, empirical formula and molecular weight of the compounds under study are shown in Table I/A. The procedures applied in their synthesis will be published elsewhere.

Toxicity tests. TAMM's method [14] was slightly modified by us. A CAM fragment was placed in 1 ml medium with or without the compound to be tested and the tubes were tightly stoppered. Each dilution of the compounds was tested in four parallel cultures. The tubes were incubated under shaking for 40 hr at 35°C. Macroscopic change in the CAM fragments was regarded as an indicator of toxic effect [14].

Antiviral activity. HORVÁTH's [13] rolling drum method was used for the determination of antiviral activity; 0.5 ml medium (containing a given dilution or none of the compound to be tested), minced CAM tissue in one drop medium and 0.1 ml virus dilution were placed in each vial. Each combination of virus and drug dilutions was tested in eight parallel cultures. The compound to be tested and the virus were added at approximately the same time. After incubation at 37°C the haemagglutinin titre of the medium was determined in TAKÁTSY's [15] Microtitrator trays. Infectivity titres were computed by the Reed-Muench formula. The antiviral effect was calculated from the difference between the control titre and the titre obtained in the presence of the drug and expressed in $\log_{10}$ units. Inhibition by 1 $\log_{10}$ unit or more has been considered significant.

The selective antiviral effect is expressed by the activity index (AI), *i.e.*, the quotient

$$AI = \frac{\text{concentration causing two-cross toxicity effect}}{\text{the lowest concentration exerting an antiviral effect}}.$$

The limit of error is estimated at $\overset{\times}{:} 2$.

Results

The antiviral effect of 25 1-aminoadamantane derivatives was examined in CAMs *in vitro*, using influenza A/H2N2/Singapore(1)57 and parainfluenza-1-(Sendai) strains as test viruses.

The structural and empirical formulae and molecular weights are presented in Tables I/A, I/B and I/C. The activity indices are shown in Tables II/A and II/B.

Discussion

The parainfluenza-1 (Sendai) virus needed a high concentration (25 μg/ml) to be inhibited in multiplication and the activity indices were too low to be used for comparison. For this reason, the effect on the antiviral activity of changes in chemical structure are discussed only in relation of the influenza A/H2N2/Singapore(1)57 strain.

A. The N-methylene aryl derivatives proved to be less active than 1-aminoadamantane hydrochloride itself. The activity of these derivatives was differently modified by the different aryl radicals.

(1) As compared to the N-benzyl derivative (H-1265) a substitution in the ortho position by an OH (H-1246) or NO_2 (H-1332) radical resulted in a 4-fold and 15-fold increase in activity, respectively. The activity index of the ortho Cl derivative (H-1275) is low owing to the high toxicity of this compound.

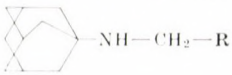
(2) The activity index of the 2-hydroxybenzyl derivative (H-1246) was reduced by substitution with a methoxy group on the ortho position (H-1333). The dimethoxy derivatives (H-1338, H-1339 and H-1340) were even less active.

(3) The activity of the Cl-substituted benzyl derivatives (H-1342, H-1275, H-1334 and H-1267) was considerably influenced by the position of the

Table I/A

Chemical characterization of the compounds

(A) Derivatives with chemical structure



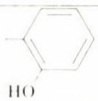
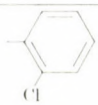
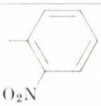
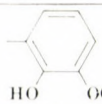
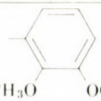
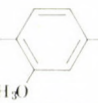
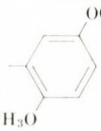
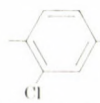
Designation	R	Empirical formula	Molecular weight
H—1265		$C_{17}H_{23}N \cdot HCl$	277.85
H—1246		$C_{17}H_{23}NO \cdot HCl$	293.84
H—1275		$C_{17}H_{22}Cl\,N \cdot HCl$	312.28
H—1332		$C_{17}H_{22}N_2O_2 \cdot HCl$	322.84
H—1333		$C_{18}H_{25}NO_2 \cdot HCl$	323.86
H—1338		$C_{19}H_{27}NO_2 \cdot HCl$	337.89
H—1339		$C_{19}H_{27}NO_2 \cdot HCl$	337.89
H—1340		$C_{19}H_{27}NO_2 \cdot HCl$	337.89
H—1342		$C_{17}H_{22}Cl\,N \cdot HCl$	312.28
H—1334		$C_{17}H_{21}Cl_2N \cdot HCl$	346.73

Table I/A continued

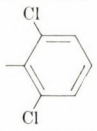
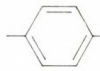
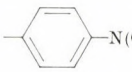
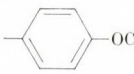
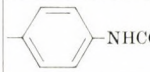
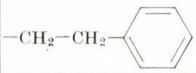
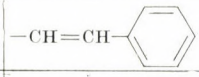
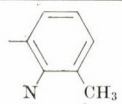
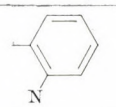
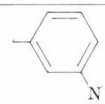
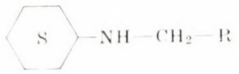
Designation	R	Empirical formula	Molecular weight
H—1267		$C_{17}H_{21}Cl_2N \cdot HCl$	346.73
H—1324		$C_{18}H_{25}N \cdot HCl$	291.86
H—1326		$C_{17}H_{22}N_2O_2 \cdot HCl$	322.84
H—1335		$C_{19}H_{27}NO \cdot HCl$	321.89
H—1250		$C_{19}H_{26}N_2O \cdot HCl$	334.89
H—1331		$C_{19}H_{27}N \cdot HCl$	305.89
H—1243		$C_{19}H_{25}N \cdot HCl$	303.88
H—1241		$C_{17}H_{24}N_2 \cdot 2\,HCl$	329.21
H—1237		$C_{16}H_{22}N_2 \cdot 2\,HCl$	315.29
H—1238		$C_{16}H_{22}N_2 \cdot HCl$	315.29
H—1239		$C_{16}H_{22}N_2 \cdot HCl$	315.29
H—1247		$C_{15}H_{21}NS \cdot HCl$	283.86

Table I/B

Chemical characterization of the compounds

(B) Derivative with chemical structure



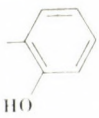
Designation	R	Empirical formula	Molecular weight
H—1264		$C_{11}H_{19}NO \cdot HCl$	241.76

Table I/C

Chemical characterization of the compounds

(C) Derivatives with chemical structure

Designation	X	Empirical formula	Molecular weight
H—1242	$HS-CH_2-COO$	$C_{12}H_{21}NO_2S$	243.37
H—1252	$HS-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-COO$	$C_{13}H_{23}NO_2S$	257.39
1-amino-adamantane HCl	Cl	$C_{10}H_{16}N \cdot HCl$	187

substituent(s), and by their relative position if more than one Cl is substituted in a molecule. The 2,6-dichlorobenzyl derivative (H-1267) had the highest activity index in view of its low toxicity.

(4) Substitution on the para position of the benzyl group (H-1324, H-1236, H-1342, H-1335 and H-1250) resulted in reduced activity as compared to H-1265.

(5) An increase in the length of the chain linking the benzyl ring with the amino group resulted in an increased toxicity and thus a low activity index (H-1265, H-1331, H-1243).

Table II/A

Activity indices of the compounds

Designation of derivative	Concentration causing two-cross toxic effect μg/ml	Lowest antiviral concentration μg/ml		Activity index	
		Influenza	Parainfluenza	Influenza	Parainfluenza
H-1265	500	5	75	100	6
H-1246	1000	2.5	100	400	10
H-1275	125	5.0	50	25	2
H-1332	750	0.5	>100	1500	<7.5
H-1333	750	15	75	50	10
H-1338	750	>20	50	<37.5	15
H-1339	250	>20	75	<12.5	3
H-1340	375	10	75	37.5	5
H-1342	180	15	25	12	7
H-1334	100	10	37.5	10	2
H-1267	2000	7.5	75	266	26
H-1324	375	10	25	37.5	15
H-1326	500	20	50	25	10
H-1335	375	>20	37.5	<18.7	10

Table II/B

Activity indices of the compounds

Designation of derivative	Concentration causing two-cross toxic effect μg/ml	Lowest antiviral concentration μg/ml		Activity index	
		Influenza	Parainfluenza	Influenza	Parainfluenza
H-1250	1000	25	200	40	5
H-1331	50	10	25	5	2
H-1243	75	7.5	25	10	3
H-1241	1500	10	100	150	15
H-1237	3000	15	100	200	30
H-1238	3000	15	200	200	15
H-1239	4000	2.5	400	1600	10
H-1247	750	2	50	375	15
H-1264	1500	25	400	60	3
H-1242	1000	0.5	100	2000	10
H-1252	1500	0.25	100	6000	15
l-aminoadamantane HCl	1500	0.5	50	3000	30

(6) Derivatives containing a heteroaromatic group (H-1241, H-1237, H-1338, H-1239 and H-1247) instead of the phenyl group were more active than the phenyl derivative (H-1265). The 4-pyridyl derivative (H-1239) was 16 times as active as H-1265.

(7) The activity index of the compound containing cyclohexyl (H-1246) instead of the adamantyl skeleton (H-1264), but the same N-substituent (N-ortho-hydroxy-benzyl) was very low.

B. The thioglycolate of 1-aminoadamantane (H-1242) was found to be as active as its hydrochloride. The AI of the mercaptopropionate of the basis (H-1252) on the other hand was twice as high as that of the hydrochloride.

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SEROLOGICAL RELATIONSHIP BETWEEN PSEUDOMONAS AERUGINOSA AND ENTEROBACTERIACEAE

II. RELATIONSHIP OF PSEUDOMONAS AERUGINOSA O ANTIGENS TO ESCHERICHIA, SHIGELLA, PROTEUS, MORGANELLA, RETTGERELLA AND PROVIDENCIA

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Summary. By agglutination and cross-absorption tests, 23 *Pseudomonas aeruginosa* strains representing all serological groups and subgroups of LÁNYI's antigenic schema were examined for relationship to 142 *Escherichia coli*, 43 *Shigella*, 59 *Proteus hauseri*, 34 *Morganella morganii*, 36 *Rettgerella rettgeri* and 61 *Providencia* O antigen type strains of international antigenic schemata. Reactions at titres 1:160 or above in at least three different serum batches for each antigen were regarded as representing a relationship. A major bilateral relationship of the a,b—a,c type was shown between *E. coli* O26 and *Ps. aeruginosa* O4a,4d and between *S. boydii* 7 and *Ps. aeruginosa* O10a,10b. Minor bilateral relationships existed between *R. rettgeri* O1a,1b, O1a,1c and *Ps. aeruginosa* O3a,3b, O3c, O3a,3d, O3a,3d,3e, O3d,3f and between *R. rettgeri* O17 and *Ps. aeruginosa* O3a,3b, O3a,3d, O3a,3d,3e, O3d,3f. More important unilateral reactions were (sera/antigens): *P. hauseri* O2/*Ps. aeruginosa* O7a,7b, O7a,7c; *P. hauseri* O20/*Ps. aeruginosa* O3a,3d,3e, O3d,3f; *Ps. aeruginosa* O4a,4b/*S. dysenteriae* 7, *S. flexneri* 1b, *S. flexneri* 4aB, *S. flexneri* 6, *S. boydii* 4, *S. boydii* 6, *S. boydii* 9; *Ps. aeruginosa* O4a,4c/*S. dysenteriae* 7, *S. flexneri* 1b, *S. flexneri* 6, *S. boydii* 9; *Ps. aeruginosa* O4a,4d/*S. dysenteriae* 7, *S. flexneri* 1b, *S. boydii* 4, *S. boydii* 9; *Ps. aeruginosa* O8/*S. flexneri* 1b, *S. flexneri* 4aB; *S. boydii* 5, *S. boydii* 9; *Ps. aeruginosa* O11/*S. flexneri* 1a, 1b, 4aB, 5, Y; *Ps. aeruginosa* O2/*P. hauseri* O22; *Ps. aeruginosa* O10a/*P. hauseri* O39; *Ps. aeruginosa* O13/*M. morganii* O4.

In a previous report [1] we described O antigenic relationships between *Pseudomonas aeruginosa* and *Salmonella–Arizona–Citrobacter*. The present paper reports on further studies of *Pseudomonas–Enterobacteriaceae* antigens.

Materials and methods

Bacterial strains. A total of 142 *Escherichia coli* international type strains represented antigens O1–O148; 43 *Shigella* strains were used as antigens for *S. dysenteriae* 1–10, provisional serotypes 3873-50, 2000-53, 3341-55, all *S. flexneri* serotypes and variants, *S. boydii* 1–15, provisional serotypes 3615-53, 2710-54 and *S. sonnei* phase 1 and 2; 59 *Proteus hauseri* strains were used for O groups and subgroups of the KAUFFMANN–PERCH schema [2, 3]; the *Morganella morganii* antigenic schema of RAUSS and VÖRÖS [4, 5] was represented by 34 type strains; the *Rettgerella rettgeri* schema of NAMIOKA and SAKAZAKI [6] by 36 type strains; for *Providencia* groups O1–O56 [7] and for groups O57–O62 [8] a total of 61 type strains were employed. The 23 *Ps. aeruginosa* type strains represented all O groups and subgroups of LÁNYI's schema [9, 10].

Immune sera were prepared in rabbits with *Enterobacteriaceae* antigens heated at 100°C for 2 ½ hr and with *Ps. aeruginosa* antigens heated at 75°C for 1 hr [9]. Sera showing homologous titres in the range 1:1280–1:10240 were used.

Tube agglutination was performed usually with bacterial suspensions heated first at 100°C for 2 ½ hr in saline then at 130°C for 1 hr in glycerol [9]. Screening examinations with *E. coli*, *M. morganii*, *R. rettgeri* and *Providencia* antigens were made with suspensions heated at 100°C for 2 ½ hr in saline. The technique of tube agglutination was described previously [1].

Results

Method of testing antigenic relationships. Bacteria heated first at 100°C, then at 130°C were suitable for demonstrating even minor relationships between *Ps. aeruginosa* and *Salmonella–Arizona–Citrobacter* [1]. The present studies with other groups of enteric bacteria confirmed this finding.

Similarly to the result of previous screenings [1], many aspecific agglutinations were recorded in *Ps. aeruginosa* sera with the *Enterobacteriaceae* antigens tested in this study and *vice versa*. These aspecific reactions appeared usually only in one batch of the serum and were in their overwhelming majority low in titre (1:20–1:80).

Antigenic relationships. Titres of 1:160 or above in at least three different batches of serum for the antigen concerned, were regarded as indicating true antigenic relationships.

Table I

Specific reactions of Ps. aeruginosa antigens in E. coli, Shigella, P. hauseri, M. morganii and R. rettgeri sera

Serum*	Homologous titre*	Titre with *Ps. aeruginosa* antigens**
E. coli O26	5120—10240	O4a,4b 40–160; O4a,4c 40–160; O4a,4d 160–1280
S. boydii 7	5120—10240	O10a 20 O10a, 10b 640–5120
P. hauseri O2	1280—5120	O7a,7b 160—320; O7a,7c 160–320
P. hauseri O20	5120—10240	O3a,3d,3e 80–160; O3d,3f 160–320
R. rettgeri O1a,1b	5120—10240	O3a,3b 320; O3c 320; O3a,3d 320; O3a,3d,3e 320–640; O3d,3f 640–1280; O13 160
R. rettgeri O1a,1c	5120—10240	O3a,3b 80–160; O3c 80–160; O3a,3d 160–320; O3d,3f 320—1280; O13 160
R. rettgeri O17	2560	O3a,3d,3e 80; O3d,3f 80–160; O13 80

* Each antigen was tested in at least 3 different batches of serum

** The antigens were heated at 100°C for 2 ½ hr, then at 130°C for 1 hr

The strains used in the experiments presented in Tables I–IV were: *E. coli* O26 F41; *S. dysenteriae* 7 9764; *S. flexneri* 1a 366/54; 1b 20013; 4aB Rabaulensis; 5 M213; 6 20048 Manchester; Y Ledingham; *S. boydii* 4 9770; 5 Yaounde 214/72; 6 9771; 7 9734 and 25006; 9 Francis 1320; *P. hauseri* O2 F392; O20 F475; O22 F233; O39 F105b; *M. morganii* O4 285; *R. rettgeri* O1a,1b T65; O1a,1c 12/261; O17 22/040; *Ps. aeruginosa* O2 170002; O3a,3b 170003; O3c 170004; O3a,3d 170005; O3a,3d,3e 170006; O3d,3f 170007; O4a,4b 170008; O4a,4c 170009; O4a,4d 170010; O7a,7b 170015; O7a,7c 170016; O8 170017; O10a 170019; O10a,10b 170020; O11 170021; O13 170023.

Table I shows the titres of *Ps. aeruginosa* antigens in sera for serologically related enteric bacteria. In Table II the reactions of *Enterobacteriaceae* antigens in *Ps. aeruginosa* sera are recorded. Four bilateral relationships are evident: a major one between *E. coli* O26 and *Ps. aeruginosa* O4a,4d, another major

Table II

Specific reactions of E. coli, Shigella, P. hauseri, M. morganii and R. rettgeri antigens in Ps. aeruginosa sera

Ps. aeruginosa serum*	Homologous titre*	Titre with *Enterobacteriaceae* antigens**
O2	5120—10240	*P. hauseri* O22 80–160
O3a,3b	2560—10240	*R. rettgeri* O1a,1b 80–160; *R. rettgeri* O1a,1c 80–160; *R. rettgeri* O17 80–160
O3a,3d	5120	*R. rettgeri* O1a,1b 80–160; *R. rettgeri* O1a, 1c 80–160; *R. rettgeri* O17 80–160
O3a,3d,3e	5120—10240	*R. rettgeri* O1a,1b 80–160; *R. rettgeri* O1a,1c 80; *R. rettgeri* O17 80–160
O3d,3f	5120—10240	*R. rettgeri* O1a,1b 80–160; *R. rettgeri* O1a,1c 80–160; *R. rettgeri* O17 80–160
O4a,4b	2560—5120	*E. coli* O26 40–160; *S. dysenteriae* 7 320–2560; *S. flexneri* 1b 320–640; *S. flexneri* 4aB 640; *S. flexneri* 6 160—320; *S. boydii* 4 160; *S. boydii* 6 80–160; *S. boydii* 9 640–1280
O4a,4c	1280—5120	*E. coli* O26 80–320; *S. dysenteriae* 7 320–640; *S. flexneri* 1b 80–320; *S. flexneri* 6 80–160; *S. boydii* 9 80–160
O4a,4d	1280—10240	*E. coli* O26 320–1280; *S. dysenteriae* 7 640–1280; *S.flexneri* 1b 80–160; *S. boydii* 4 80–160; *S. boydii* 9 320
O8	1280—2560	*S. flexneri* 1b 1280–2560; *S. flexneri* 4aB 640–1280; *S. boydii* 5 640–1280; *S. boydii* 9 320–640
O10a	1280—10240	*S. boydii* 7 2560–5120; *P. hauseri* O22 80–160
O10a,10b	1280—10240	*S. boydii* 7 1280–5120
O11	5120	*S. flexneri* 1a 1280; *S. flexneri* 1b 640–1280; *S. flexneri* 4aB 320–640; *S. flexneri* 5 80–320; *S. flexneri* Y 320–640
O13	1280—2560	*M. morganii* O4 640–1280

* Each antigen was tested in at least 3 different batches of serum
** The antigens were heated at 100°C for 2 ½ hr then at 130°C for 1 hr

one between *S. boydii* 7 and *Ps. aeruginosa* O group 10, and two minor relationships between *R. rettgeri* group O1 and *Ps. aeruginosa* group O3 and between *R. rettgeri* group O17 and certain subgroups of *Ps. aeruginosa* O3. The rest of the reactions indicated unilateral relationships, although some of them were remarkable in titre. From Tables I and II, the reactions obtained in *Providencia* O11, O30 and O48 sera were omitted. Different batches of these sera agglutinated several *Ps. aeruginosa* cultures (O1, all O3, all O4, O11 and O12) to low titre (1:40–1:160).

Table III shows the antigenic analysis of *E. coli* O26—*Ps. aeruginosa* O4a,4d relationship. From agglutination titres given by heated cultures it is obvious that each of the two organisms has a main factor lacking in the other, while the common antigen is a less important factor in both strains. It may be concluded from the low titre cross-agglutinations between *E. coli* O26 and

Table III

Analysis of the relationship between E. coli O26 and Ps. aeruginosa O4a, 4d

Antigen	Serum				
	E. coli O26 unabsorbed	*E. coli* O26:K60(B6) unabsorbed	*E. coli* O26 absorbed by *Ps. aeruginosa* O4a, 4d	*Ps. aeruginosa* O4a, 4d unabsorbed	*Ps. aeruginosa* O4a, 4d absorbed by *E. coli* O26
E. coli O26 heated*	5120	10240	2560	640	0
E. coli O26:K60(B6) living	0	320 K-type	0	80K-type	0
Ps. aeruginosa O4a,4d heated*	160	640	0	10240	1280
Ps. aeruginosa O4a,4d living	0	320	0	10240	1280

* 100°C for 2 ½ hr + 130°C for 1 hr

Ps. aeruginosa O4a,4b and O4a,4c that the common factor is probably a part of *Ps. aeruginosa* antigen O4a. *Ps. aeruginosa* living culture failed to agglutinate in *E. coli* serum O26 (prepared with heated antigen), but gave a strong, although low-titre reaction in serum *E. coli* O26:K60(B6) (prepared with alcoholized antigen). Living *E. coli* O26:K60(B6) suspension showed a low titre K-type (coarse granular) agglutination in *Ps. aeruginosa* serum O4a,4d.

Table IV

Analysis of the relationship between S. boydii 7 and Ps. aeruginosa O10

Antigen	Serum						
	S. boydii 7 unabsorbed	*S. boydii* 7 absorbed by *Ps. aeruginosa* O10a	*S. boydii* 7 absorbed by *Ps. aeruginosa* O10a,10b	*P. aeruginosa* O10a unabsorbed	*Ps. aeruginosa* O10a absorbed by *S. boydii* 7	*Ps. aeruginosa* O10a,10b unabsorbed	*Ps. aeruginosa* O10a,10b absorbed by *S. boydii* 7
S. boydii 7	10240	10240	320	2560	0	5120	0
Ps. aeruginosa O10a	20	0	0	5120	80	10240	160
Ps. aeruginosa O10a,10b	5120	5120	0	5120	80	10240	320

Table IV presents the results of cross-absorption test between *S. boydii* 7 and *Ps. aeruginosa* group O10 type strains. The relationship of the former to *Ps. aeruginosa* O10a,10b is a bilateral, a, b—a, c type one. To *Ps. aeruginosa* O10a, *S. boydii* 7 is related in a unilateral manner: in serum O10a *S. boydii* 7 shows a considerable titre, but in serum *S. boydii* 7 culture O10a practically fails to agglutinate. That the relationship involves not only antigen O10b.

is shown also by the fact that after absorption of serum O10a by *S. boydii* 7 there is a great decrease in antibody titre against strain O10a.

Absorption experiments with other related antigens gave the following results. The unilateral connections of *Ps. aeruginosa* groups O4 and O8 to shigellae were due to several different minor factors, since absorption of the corresponding *Ps. aeruginosa* sera with one reacting *Shigella* culture usually failed to remove agglutinins for the others. From *R. rettgeri* O1a,1b and O1a,1c sera any *Ps. aeruginosa* O3 culture absorbed agglutinins for other members of the group, except for O3d,3f which seemed to share an additional factor with *R. rettgeri* group O1.

Discussion

The results of the present study are in accordance with our previous findings that few real antigenic relationships exist between *Ps. aeruginosa* and *Enterobacteriaceae*. Agglutinations recorded in the course of screening examinations were mostly aspecific ones associated with antibodies not specific for antigens of the immunizing culture.

As to the reactions confirmed in different batches of sera, it would appear that antigens responsible for the intragroup relationship of a given species, were usually involved in the interfamiliar relationship (*Ps. aeruginosa* O3a, O4a and O7a, *R. rettgeri* O1a, *S. flexneri* group factors).

As in previous studies on the relationships between *Ps. aeruginosa* and *Salmonella–Arizona–Citrobacter* antigens, part of the reactions between organisms examined in these experiments were unilateral ones. In this respect *Ps. aeruginosa* group O10 behaved unusually, since the relationship of *S. boydii* 7 to O10a was unilateral, while that to O10a,10b was bilateral (Table IV).

Wiedermann and Flamm [11] showed that certain *Ps. aeruginosa* isolates agglutinated in *E. coli* O26:K60(B6) serum and that these cultures were antigenically related to the corresponding *E. coli* serogroup. The present studies have confirmed this observation: in working dilution of serum *E. coli* O26:K60(B6) *Ps. aeruginosa* O4a,4d cultures gave a fairly definite slide agglutination and the same result was obtained by tube agglutination with either heated or living suspensions. In view of the fact that on media used for the isolation of enteropathogenic coliforms, some *E. coli* O26:K60(B6) strains fail to produce lactose-fermenting colonies [12] and that *Ps. aeruginosa* O4a,4d is the serogroup which occurs most frequently in the faeces of infants [9], this finding is of some diagnostic importance.

The relationship between *E. coli* O26 and *Ps. aeruginosa* O4a,4d is also of theoretical interest. In *E. coli* O26 serum (prepared with heated culture) the agglutination of living cells of the homologous strain is inhibited owing to the supposed masking effect of a factor termed K antigen. In *Ps. aeruginosa* no K-type antigens have been observed [9], yet the living *Ps. aeruginosa*

culture failed to agglutinate in *E. coli* O26 serum. The living *E. coli* culture gave a K-type (coarse granular and relatively low titre) agglutination not only in serum *E. coli* O26:K60(B6) but also in serum *Ps. aeruginosa* O4a,4d. From this finding it would appear that the factor responsible for the *Ps. aeruginosa–E. coli* relationship is present not only in antigen O26, but also in antigen K60 (B6), and that in the former it behaves as an O antigen (thermoresistant) and in the latter as a K antigen (which fails to agglutinate in O serum).

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POSSIBLE ROLE OF ADENOSINE DEAMINASE IN SUBOPTIMAL GROWTH OF THE BACILLUS ANTHRACIS ADENINE AUXOTROPH MUTANT

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Summary. The adenyl succinate lyase (EC 4. 3. 2. 2) deficient *Bacillus anthracis* mutant showed weaker growth than did the prototroph despite the presence in the medium of appropriate concentrations of adenine or adenosine. The rate of ATP synthesis was also lower as compared to the prototroph. Adenosine deaminase (EC 3. 5. 4. 4) and nucleoside phosphorylase (EC 2. 4. 2. 1) were demonstrated in the extracts of both strains. In the auxotroph mutant, adenosine deaminase reduced the synthesis of nucleotide from exogenous adenosine and adenine. The fact that the adenine nucleotide pool of the mutant was below the physiological level, might explain the suboptimal growth ability, *viz.* the avirulent nature of the mutant.

IVÁNOVICS and his team prepared several well defined purine, pyrimidine and vitamin auxotroph mutants from the acapsulogenic Vollum strain of *Bacillus anthracis* [1–3]. The isolated mutants were classified on the basis of alkaline phosphatase production [4], among others. Among the purine requiring auxotrophs the capsulogenic variants of adenyl succinate synthetase and adenyl succinate lyase enzyme deficient mutants produced toxin, but proved avirulent in mice [5]. Alkaline phosphatase synthesis by both adenine requiring mutants was partially de-repressed [4]. Accumulation of alkaline phosphatase in the area between the cytoplasmic membrane and the cell wall was observed in the adenyl succinate lyase deficient strain. The accumulated enzyme caused injury of the cell wall [6] probably through splitting of the organic phosphate compounds which participate in the mural architecture. The cell wall of this mutant, *viz.* of the prototroph strain, did not contain the phosphorus-containing polymer, teichoic acid, which is a characteristic component of the wall of most Gram-positive bacteria [7]. Experimental observations suggested the responsibility of the low level of adenine nucleotide pool for the de-repressed alkaline phosphatase production of the adenyl succinate lyase deficient mutant on the grounds that, apart from inorganic orthophosphate it was repressible by adenine nucleotides [8]. Even subtoxic amounts of adenine failed to promote growth of the mutant *in vivo* [2]. On the basis of these findings it was assumed that the non-pathogenic nature of the adenine auxotroph strain chiefly depended on the suboptimal adenine nucleotide synthesis. The present paper is a report of studies on factors influencing the growth of the mutant *in vitro*.

Materials and methods

Bacterial strains. B. anthracis Vollum non-capsulogenic prototroph, designated as VR. Of the *B. anthracis* Vollum non-capsulogenic adenine auxotroph mutants, the 23C⁻ ade⁻ adenyl succinate lyase deficient strain was used.

Media. The basal medium containing lactalbumin hydrolysate was completed by appropriate amounts of salts, glucose, vitamin B_1, adenine and K_2HPO_4 [4]. From the lactalbumin hydrolysate, before its addition to the medium all phosphorus were removed by barium hydroxide [7].

Cultivation. The inorganic orthophosphate concentration of the lactalbumin medium was made up to 1.0 mM and its adenine concentration was completed with 10—200 μg/ml adenine, depending on the purpose of the experiment. Ten ml amounts of the medium thus completed were distributed into 100 ml Erlenmeyer flasks and 10^5 spores were inoculated per flask. Incubation was done in a reciprocal shaker in a water bath of 37 °C under aeration for 16 hr.

Determination of optical density. This was carried out in a Magnephot II (Orion, Hungary) densitometer.

Protein determination was done in *B. anthracis* cultures disintegrated by sonication, by the method of LOWRY *et al.* [9]. The colour intensity of the samples was determined at 750 nm in a Spectromom 201 (MOM, Hungary) spectrophotometer. Bovine albumin 400—800 μg/ml dissolved in 0.1 N sodium hydroxide was used as reference standard.

Determination of adenosine triphosphate. Different quantities (10, 50 and 100 μg/ml) of adenine were added to the basal medium containing 1.0 mM orthophosphate, into which equal numbers of spores from the prototroph and adenine auxotroph mutants were inoculated. From the cultures, samples of 2.0 ml were withdrawn every hour and 2.0 ml of 0.5 N ice cold perchloric acid was added to each sample. The cooled samples were placed in a melting ice bath for 30 min and centrifuged. The supernatants were neutralized by the addition of 10 N K_2CO_3 [10]. The potassium perchlorate precipitate was removed by centrifugation and the concentration of ATP in the supernatants was estimated by luciferase [11].

A 5.0 mg/ml solution of luciferase (Firefly Lantern extract, Serva, Heidelberg, GFR) was prepared in distilled water. For ATP determination, 0.4 ml distilled water and 0.1 ml enzyme solution, then 0.1 ml bacterium extract were pipetted into the cuvette. Measurement was begun 15 s after the addition of the bacterial extract in a Packard Tri-Carb liquid Scintillation Spectrometer Model 2001. Scintillation counts were recorded for 1 min. Various dilutions of 5×10^{-4} mμM/ml ATP stock solution were added to the enzyme in 0.1 ml amount as reference standard.

Preparation of crude bacterial extract. Four-hundred ml each from 16 hr cultures of the prototrophic and adenine auxotrophic bacterium were centrifuged in the cold. The medium was completed with 50 μg/ml adenine for cultivation of the mutant. The sediment was resuspended in 0.1 M cacodylate buffer pH 7.4, adjusted to OD 1.0 and centrifuged. The second sediment was resuspended in one tenth of the previously used volume. Five ml of the suspension were disintegrated in the cold for 4×1 minutes in a MSE Ultrasonic Power Unit (MSE, England). The sonicated bacterial suspension was centrifuged for 10 min at 1000 rpm and the enzyme activity of the supernatant was determined.

Determination of adenosine deaminase (EC 3. 5. 4. 4). The bacterial extract prepared as above contained 2.5 mg/ml protein. The system used for the measurement of enzyme activity contained 25 μl bacterium extract and 200 μl substrate in 3.0 ml 0.1 M cacodylate buffer of pH 7.4. Changes in adsorption were determined in the samples at one-minute intervals at 256 nm, in another experiment at 240 nm, at 37 °C, in a UNICAM SP 800 spectrophotometer. Adenosine, deoxy-adenosine and AMP were used as substrates.

The decomposition (de-amination) of adenosine was assessed from the absorption values measured at 265 nm, the amount of inosine formed from those measured at 240 nm [12].

Metabolism of ^{14}C adenine. The extract of the prototrophic cells contained 1.52 mg/ml protein, that of the adenine auxotrophic cells 1.71 mg/ml protein. From both suspensions, 0.4 ml was transferred into small tubes and 1 μCi ^{14}C adenine (140 mμM, Amersham Laboratories, England) was added in 100 μl volume. The tubes were incubated in a water bath at 37 °C, 10 μl samples were withdrawn at 10-min intervals and chromatographed in silica gel. The silica gel GF_{245} (Merck) plates, 10×20 cm, were activated for 30 min at 120 °C, then 5 μg each from cold adenine, adenosine, hypoxanthin and inosine were applied to starting points to serve as control. Ten μl from each sample were then applied to the starting points of the plates prepared as above. A mixture of n-butanol, ethyl acetate, methanol and 25% ammonium hydroxide (14 : 8 : 6 : 8) was used as running solvent [13]. After drying, the spots were detected by an UV lamp and marked (R_F: adenine, 0.68; adenosine, 0.57; hypoxanthine, 0.45; inosine,

0.23). The marked spots and start spots were scraped off separately and each was suspended in 5.0 ml scintillation fluid consisting of 0.5 g POPOP and 5.0 g PPO in 1.0 litre toluene. The activity of each sample was measured in a Packard Tri-Carb liquid Scintillation Spectrometer, Model 2001 for 1 min.

Results

Growth of the prototroph and adenine auxotroph mutants of *B. anthracis* was studied under different conditions. The bacteria were cultivated in the presence of inorganic orthophosphate at various concentrations and growth was expressed in terms of rising optical density. It was shown in previous experiments that the alkaline phosphatase production of both strains was markedly de-repressed in the presence of 0.1 mM phosphate. Phosphate at 1.0 mM concentration repressed enzyme production by the prototroph to a minimum and 2.0 mM repressed also that of the auxotroph [4, 8].

The growth medium contained a minimum amount (0.1 mM) of orthophosphate which was completed with various amounts of adenine for cultivation of the mutant.

The degree of mutant growth did not reach that of the prototroph even at the applied maximum adenine concentration of 100 μg/ml. In further experiments, orthophosphate concentration in the medium was completed to 1.0 or 2.0 mM.

Growth of the auxotroph did not reach the degree shown by the prototroph either with an elevated concentration of phosphate, or with an elevated concentration of adenine. Increase of the adenine concentration to 200 μg/ml even inhibited growth.

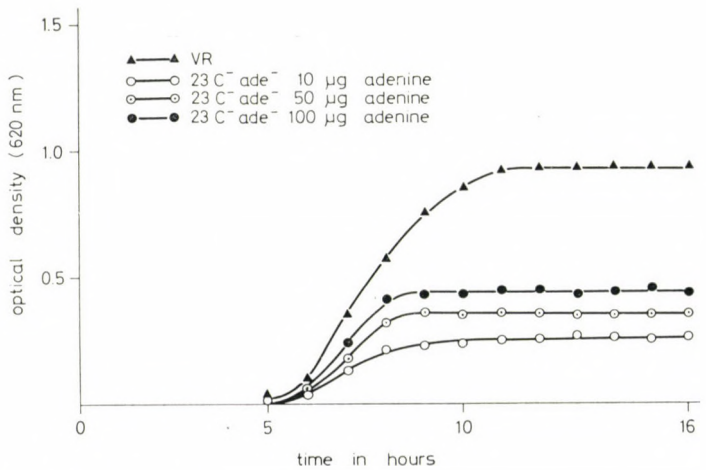

Fig. 1. Growth of *B. anthracis* prototroph (VR) and adenine auxotroph (23 C^- ade^-) in 0.1 mM orthophosphate containing medium, in the presence of adenine at various concentrations

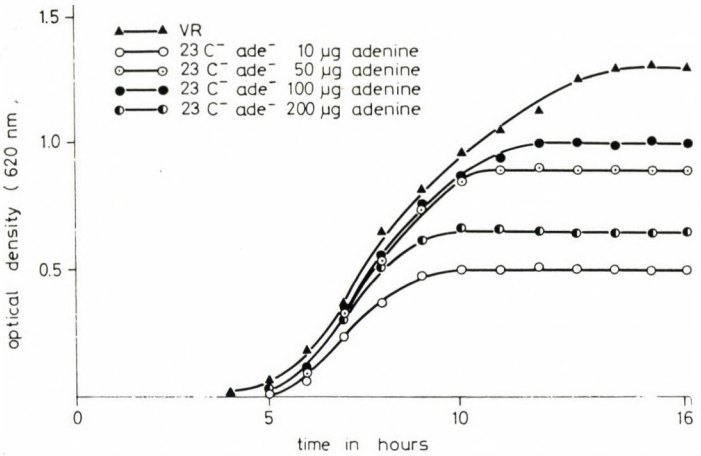

Fig. 2. Growth of *B. anthracis* prototroph (VR) and adenine auxotroph (23 C⁻ ade⁻) in 1.0 mM orthophosphate-containing medium, in the presence of adenine at various concentrations

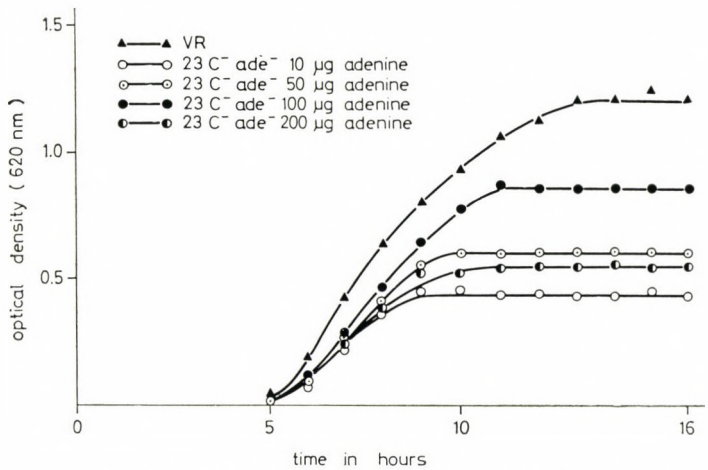

Fig. 3. Growth of *B. anthracis* prototroph (VR) and adenine auxotroph (23 C⁻ ade⁻) in 2.0 mM orthophosphate-containing medium, in the presence of adenine at various concentrations

As the concentration increase of both adenine and phosphate in the medium also failed to promote auxotroph growth to the degree found with the prototroph, ATP synthesis was examined for dependence on the applied concentrations of adenine.

The quantity of ATP synthesized by the mutant increased and the degree of its decomposition decreased with the rise of adenine concentration in the medium. At 50 μg/ml adenine concentration, the auxotroph synthesized the same amount of ATP as did the prototroph. Equal concentrations of adenine, adenosine, deoxy-adenosine, AMP and ATP were subsequently tested, each in itself, for effect on mutant growth.

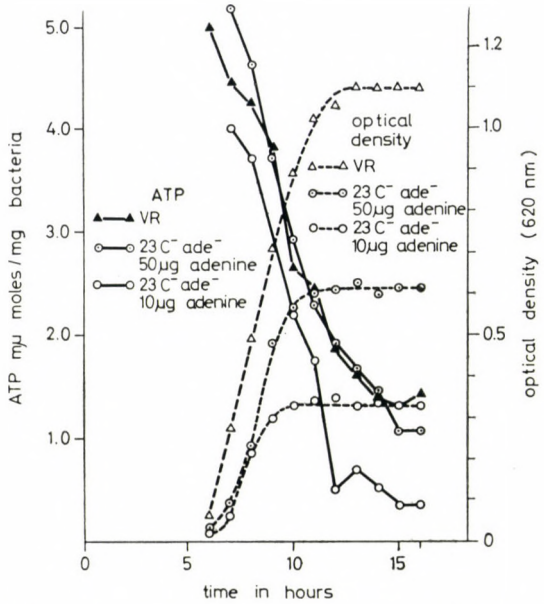

Fig. 4. Changes of ATP synthesis in *B. anthracis* prototroph (VR) and adenine auxotroph (23 C^- ade^-) mutants in the function of time, at various concentrations of adenine. The medium contained 1.0 mM orthophosphate

Table I

Growth of the B. anthracis adenine auxotroph mutant in the presence of adenine, adenosine, deoxyadenosine, AMP and ATP at different concentrations

Substrate concentration in the medium mM		Optical density
Adenine	0.05	0.20
	0.10	0.40
	0.20	0.52
	0.30	0.60
	0.40	0.68
Adenosine	0.05	0.06
	0.10	0.11
	0.20	0.24
	0.30	0.32
	0.40	0.39
Deoxyadenosine	0.40	0.02
AMP	0.40	0.01
ATP	0.40	0.03

The medium contained 1.0 mM inorganic orthophosphate. Optical density of the samples was determined after 16 hr incubation.

In these experiments, bacterial growth was expressed in terms of optical density, because the mutant multiplied differently in the presence of the various substrates. The auxotroph strain showed optimum growth in the presence of adenine; nucleosides were utilized by it to a lesser degree and nucleotides practically not at all. The weaker growth in the presence of adenosine as compared to an equal concentration of adenine may have been due to the weaker permeation capacity of adenosine and suggested the possibility of its partial breakdown or de-amination. To obtain more information on this problem, a sonicated cell suspension of the auxotroph mutant was tested for its effect on the substrates adenine, adenosine, deoxyadenosine and AMP.

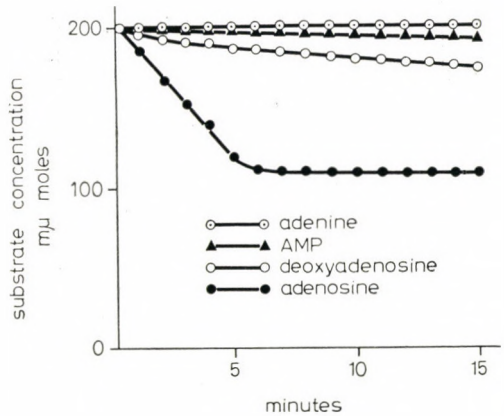

Fig. 5. Substrate specificity of adenosine deaminase in cell extract from *B. anthracis* adenine auxotroph mutant. To 3.0 ml bacterial extract, containing 65 μg/ml protein, 200 mμM adenine, adenosine, deoxyadenosine or AMP were added. The quantitative decrease of the substrates was determined at 265 nm in the function of time

The bacterial extract had no influence on adenine, but de-aminated 10 mμM of AMP, 30 mμM of deoxyadenosine and 90 mμM of adenosine during 15 min incubation. Subsequently, the crude bacterial extract was tested for adenosine deaminase activity.

The amount of adenosine decreased by 80 mμM, whereas that of inosine rose to 75 mμM during the period of incubation. The cell extract of the adenine auxotroph mutant was tested for adenosine deaminase activity in the presence of various substrate concentration.

The *Km* value of the enzyme was calculated from the substrate saturation curve according to LINEWEAVER and BURK [14]. The Michaelis constant M proved to be 3.3×10^{-5} per litre.

Since the photometric study of adenine metabolism was limited by the high protein content of the bacterial extract, ^{14}C adenine metabolism was next examined by chromatography, as described above. A considerable amount of free adenine was still present after 60 min of incubation of the adenine re-

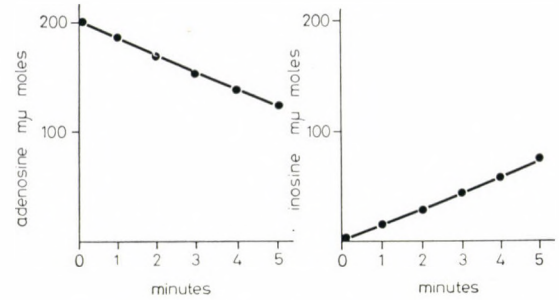

Fig. 6. Testing of *B. anthracis* adenine auxotroph cell extract for adenosine deaminase activity. To 3.0 ml of bacterial extract, containing 65 μg/ml protein, 200 mμM adenosine was added. The quantitative decrease of adenosine was followed up at 265 nm, the increase of inosine concentration, at 240 nm

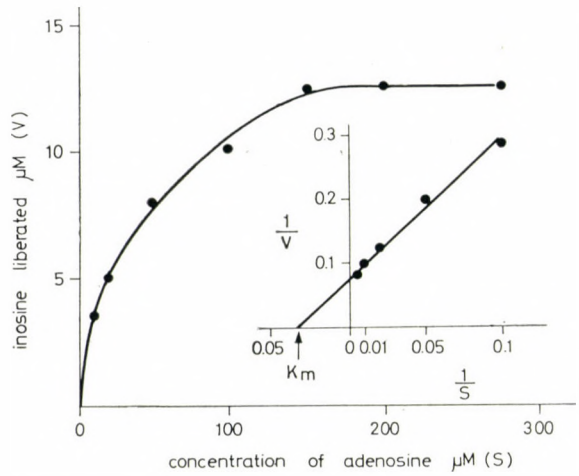

Fig. 7. Adenosine deaminase activity of *B. anthracis* adenine auxotroph cell extract at rising substrate concentration. Different amounts of adenosine were added to 3.0 ml cell extract, containing 27 μg/ml protein. The samples were incubated at 37 °C for 5 min and the quantities of inosine formed were determined at 240 nm

quiring strain. The concentrations of labelled inosine and hypoxanthine rose in the function of time as adenine decreased.

The sonicated cell suspension of the prototroph strain produced a greater decrease of the added adenine concentration under similar conditions. Adenine either disappeared or fell to a minimal level during 40 min incubation, whereas the hypoxanthine formed slightly exceeded the amount of inosine.

These findings suggest that several enzymes play a role in the adenine metabolism of *B. anthracis*.

It has been concluded that the transformation of labelled adenine to hypoxanthine took place in at least three steps: first, adenine was transformed

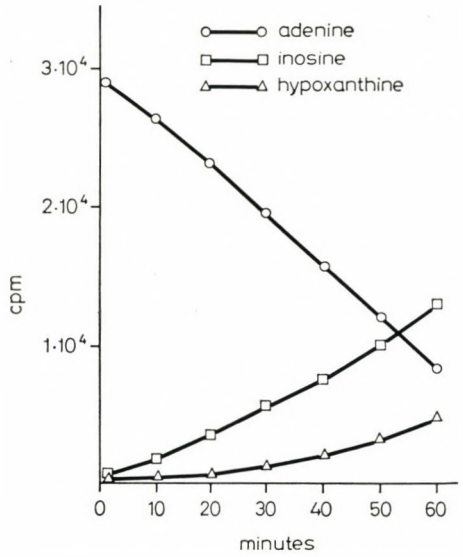

Fig. 8. Influence of *B. anthracis* adenine auxotroph cell extract on metabolism of ^{14}C adenine. To 400 μl cell extract, containing 1520 μg/ml protein, 100 μl labelled adenine was added and samples of 10 μl were chromatographed at different points of time. The cpm values are related to 10 μl samples

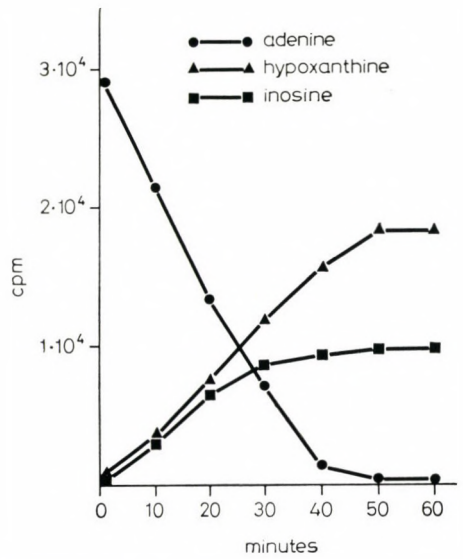

Fig. 9. Influence of *B. anthracis* prototroph cell extract on metabolism of ^{14}C adenine. To 400 μl cell extract, containing 1710 μg/ml protein, 100 μl labelled adenine was added and samples of 10 μl were chromatographed at different intervals. The cpm values are related to 10 μl samples

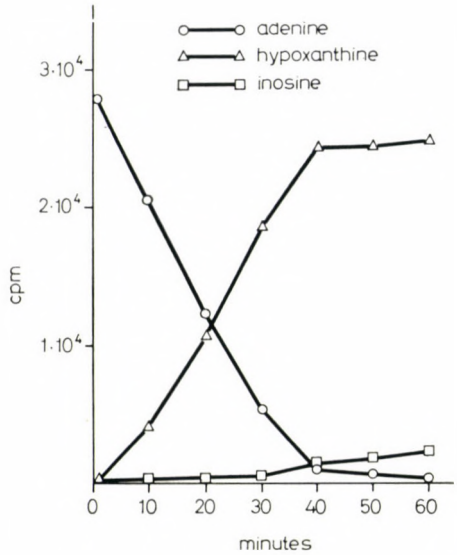

Fig. 10. Influence of *B. anthracis* adenine auxotroph live cell suspension on the metabolism of ^{14}C adenine. To 400 μl cell suspension, containing 2110 μg/ml protein, 100 μl labelled adenine was added and samples of 10 μl were chromatographed at different intervals. The cpm values are related to 10 μl samples

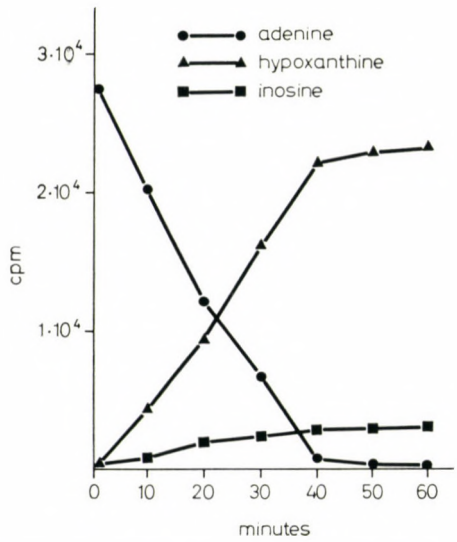

Fig. 11. Influence of *B. anthracis* prototroph live cell suspension on the metabolism of ^{14}C adenine. To 400 μl cell suspension, containing 2025 μg/ml protein, 100 μl labelled adenine was added and 10 μl samples were chromatographed after different periods of incubation. The cpm values are related to 10 μl samples

into adenosine, which gave rise to inosine by de-amination and finally hypoxanthine arose from inosine, probably under the influence of nucleoside phosphorylase.

It should be noted that equal amounts of live (non-sonicated) cells in suspension of the prototroph or adenine auxotroph strain metabolized ^{14}C adenine in a nearly similar degree under the experimental conditions employed for cell suspensions disintegrated by sonication.

Much less inosine but more hypoxanthine was formed in the function of time than in the case of the disintegrated cell suspensions.

Discussion

Many authors have studied the auxotroph mutants of pathogenic bacteria and a relationship was sought between requirements and virulence. Observations on the virulence of the purine requiring mutants of *Pseudomonas pseudomallei* [15], *Salmonella typhi-murium* and *Salmonella typhi* [18] were, however, particularly contradictory. This was probably due to the lack of information concerning the precise site of the block in purine synthesis. IVÁNOVICS *et al.* [2] have shown that virulence is determined by the purine requirement if the conversion of inosinic acid to adenylic acid does not take place. They also found that a lack of the synthesis of adenyl-succinate synthetase or adenyl succinate lyase was responsible for the loss of virulence by *B. anthracis* adenine auxotroph mutants. It was postulated that apart from adenine deficiency, other factors, thus probably changes in the cell wall architecture, play a role in the mutant's loss of virulence for mice. The adenine requiring mutants failed to multiply in the animal despite administration of the base in subtoxic doses, although a satisfactory blood level of adenine could temporarily be ensured. Although free guanine does not occur in the abdominal cavity of the animal, the guanine-deficient mutants began to multiply at a slower rate than the prototroph did, but finally they killed the animal [2].

Attempts to explain the avirulent nature of the adenine requiring mutants have led to the conclusion that several factors must play a role in the phenomenon. Although adenine is rapidly eliminated by the animal [19, 20], a satisfactorily high blood level can temporarily be maintained [2]. But this in itself was not sufficient, as the adenine auxotroph mutant failed to display optimum growth even under well-controlled conditions *in vitro*. An increase of the adenine concentration in the medium above a given level inhibited rather than promoted the mutant's growth. It has been shown that an adenine-specific feed-back inhibition takes place in the adenyl succinate lyase deficient mutants of *Escherichia coli* and *S. typhi-murium* [21]. Of the factors playing a role in the avirulence of the *B. anthracis* adenine auxotroph mutant, the

following could be identified on the basis of the present experiments. In the presence of 10 μg/ml adenine, ATP synthesis by the mutant was lower than that by the prototroph. The growth requirements of the mutant could also be satisfied with adenosine, although it failed to utilize the nucleoside to such an extent as it utilized the base.

It was also found that the mutant develops an adenosine deaminase activity, which may influence the synthesis of adenine nucleotides. Epxeriments with ^{14}C adenine showed that adenosine deaminase indirectly participated in the adenine metabolism of the mutant. The presence of hypoxanthine may be attributed to the strains' nucleoside phosphorylase. Other authors [22, 23] demonstrated adenosine deaminase in *Bacillus cereus*, which is phylogenetically closely related to *B. anthracis*. Consideration should also be given to the recent electron microscopic finding that the de-repressed alkaline phosphatase synthesis of the *B. anthracis* adenine auxotroph may alter the structure of the bacterial cell wall, a circumstance that probably decreases bacterial resistance against the host's protective mechanism [6].

Acknowledgement. The authors are indebted to Professor G. Iványovics for valuable advice in the experiments and for help in the preparation of this report.

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MACROPHAGE MIGRATION INHIBITION AS AN IN VITRO CORRELATE OF CUTANEOUS DELAYED SKIN REACTION

I. METHODICAL CONSIDERATIONS

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Summary. For the investigation of delayed type hypersensitivity the macrophage migration inhibition technique has been introduced with some modifications. Qualitative analysis of the peritoneal exudate cells showed that monocyte-macrophages were present in 60—98%, lymphocytes in 1—24%, eosinophil leucocytes in 0—11%. Average control chamber growth of untreated and BCG vaccinated animals displayed an average value of 95 and 80 mg, respectively (from 7 to 256 mg). The within animal SD was estimated in 5 cases yielding ±33% of the control value. The necessity of determining the highest non-toxic antigen dose for the measurement of macrophage migration inhibition of sensitized animals is suggested.

In the course of investigations into intracellular parasitism, examination of the development of cellular immunity has assumed importance. Therefore, the recently elaborated macrophage migration inhibition test [1–3] has been introduced in our laboratory as an *in vitro* correlate of cellular immunity. The present paper is reporting on experience gained by the application of this method.

Materials and methods

Preparation of peritoneal exudate cells (PEC). Guinea pigs of both sexes weighing 250—300 g were used throughout. Older animals (weighing 600—800 g) did not yield PEC in a sufficient quantity. The guinea pigs received 30 ml sterile mineral oil intraperitoneally and were bled on the 5th day following the injection. PEC were collected by washing the peritoneal cavity through a median incision with about 60 ml sterile Hanks' solution. Blood-tinged exudates were discarded. The PEC were washed twice with sterile TC 199 solution. A smear prepared from PEC was stained by Pappenheimer's dye for qualitative analysis. The cell suspension was kept in a refrigerator until use.

Test procedure. Following the last centrigufation the supernatant was carefully decanted while retaining a small remnant which did not exceed 1/4—1/3 of the cell volume. PEC were thoroughly mixed in this fluid with a glass rod and a capillary 0.7—0.8 mm in diameter was let to fill with the cell suspension by capillarity. An adequate dilution of the cell suspension was evident when its borderline slowly climbed up the capillary. Mixing of PEC was repeated every time when a new capillary was used. In this manner the laborius centrifugation of capillaries could be omitted. The control and antigen chambers for the same animal were always prepared from the same capillary.

At the beginning, Mackaness type chambers were used for cell culturing, later a more simple set has been developed (Fig. 1) which contained 6 holes 12 mm in diameter and of 0.25 ml content each. These plates could be very skilfully used for the simultaneous preparation of 6 parallels. The control chambers were filled with TC 199 containing 15% normal guinea pig serum. A due quantity of the antigen was added to this culturing fluid for the measurement of migration inhibition. The capillaries containing PEC were broken into pieces of 5—6 mm

length, sealed and simultaneously fixed on cover glasses with hot paraffin. Following incubation at 37 °C for 24 hr, the growth of PEC was projected on paper of uniform thickness, traced, cut and the paper was weighed on an analytical balance. Data of migration inhibition are given according to the following formula:

$$100 = \frac{\text{average growth in antigen chamber}}{\text{average growth in control chamber}} \times 100$$

Macrophage migration was investigated in control and in BCG vaccinated ($10^4 - 10^5$ viable units) guinea pigs.

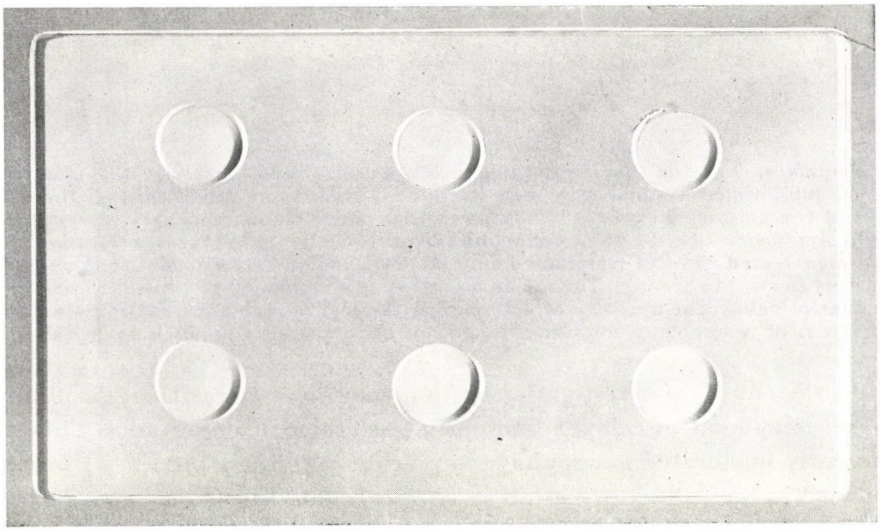

Fig. 1. Simple set for the preparation of 6 parallel macrophage migration chambers

Results

Qualitative analysis of 115 peritoneal exudates obtained from control and BCG vaccinated guinea pigs revealed among PEC 81.6% (60–98%) monocyte-macrophages, 5.6% (1–24%) lymphocytes, and 0.9% (0–11%) eosinophils. Disintegrated monocytes could be found in 7.8% (0–30%). In two cases the number of disintegrated cells reached about 50% without any apparent change in control macrophage growth. This would suggest that the destruction could be the consequence of the preparation of smears. There was no considerable difference in cellular composition between the PEC of control ($n = 30$) and BCG vaccinated ($n = 85$) guinea pigs.

A high variability of control chamber growth between animals was observed in agreement with several authors [1, 4, 5] though most papers mention only per cent inhibition and not the measured individual values for macrophage migration. The calculated mean was in our experiments 95 mg in the case of controls and 80 mg in the case of BCG vaccinated guinea pigs (4 mm^2 area

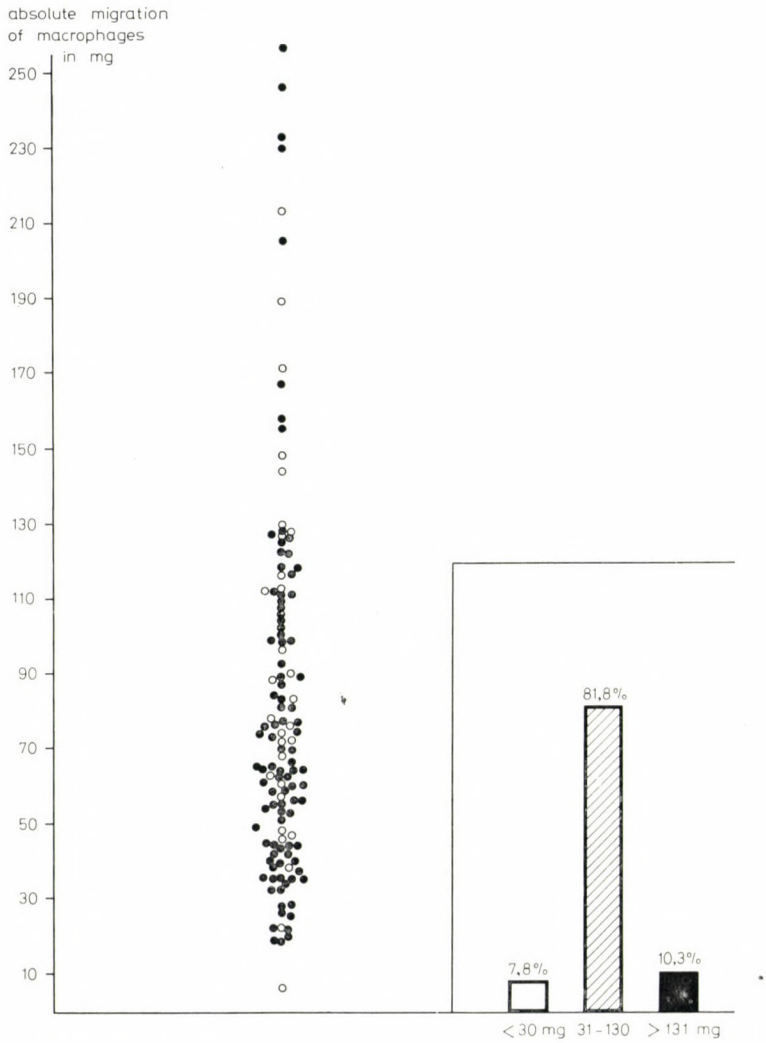

Fig. 2. Absolute migration of macrophages in antigen-free nutrient medium. Open circles = untreated guinea pigs; closed circles = BCG vaccinated guinea pigs. Insert: per cent of low (clear column), medium (shaded column) and high (solid column) values for control macrophage migration

corresponds to 47 ± 3.9 mg). The lowest observed value was 7 mg and the highest 256 mg (Fig. 2).

The majority (82%) of the data ranged between 30–130 mg and only 10.3% were above and 7.8% below these values. Exceptionally high or low values were unfrequent. It should be mentioned that low control values present a great difficulty in the evaluation of macrophage migration inhibition.

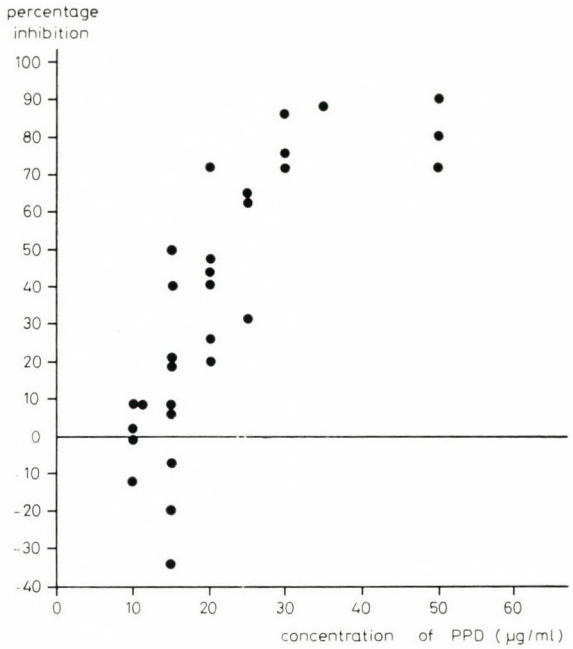

Fig. 3. Titration of the non-toxic PPD dose on control macrophages

Variability of macrophage growth in different control chambers of the same animal is also considerable. The within animal SD of 9–10 parallels was estimated in 5 cases; these yielded an SD of $\pm$37.1; $\pm$15.0; $\pm$19.1; $\pm$52.2; $\pm$50.8 mg, *i.e.*, 35, 26, 41, 24, and 37% (in average, 33%) of the control. In view of this, at least 6 parallels were used routinely.

Migration inhibition was investigated in BCG treated animals with PPD (Statens Serum Institute, Copenhagen), as specific antigen. A PPD concentration of 30 μg/ml, though widely used [6—9], was too high under our experimental conditions because macrophage migration was inhibited not only in BCG vaccinated animals but also in the control ones. Therefore the *in vitro* toxicity of PPD on control PEC was examined (Fig. 3). A dose of 50 μg/ml PPD caused complete migration inhibition (81%), the slope was very steep and only 10 and 15 μg/ml PPD proved to be ineffective on control macrophage growth. According to these data, all investigations were made by applying the highest non-toxic PPD doses.

The question arose whether the applied doses were really non-toxic ones, or there was a spurious lack of effect in consequence of the equilibrium of stimulatory and inhibitory effects on macrophage growth [10]. No stimulatory effect of PPD could be demonstrated at concentrations lower than 10 μg/ml (Fig. 4).

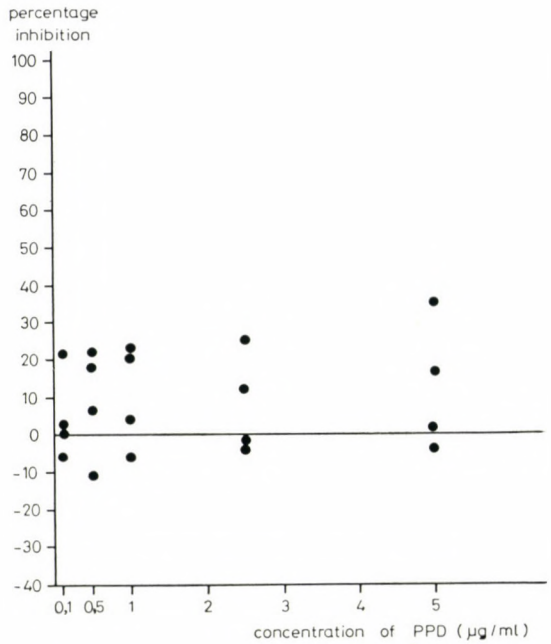

Fig. 4. Effect of low PPD concentrations on control macrophages

Discussion

The macrophage migration technique has gained world-wide acceptance in experimental [11–21] and clinical [22–30] work for the demonstration *in vitro* of delayed hypersensitivity. HUGHES [31], BERGSTRAND and KÄLLEN [32] and MAINI *et al.* [33] have pointed to the limitations and difficulties of the macrophage migration test. Among these difficulties the most severe is the considerable variability of macrophage growth in control chambers. According to LAMELIN [34] "Everyone who has used the technique realizes that even under identical conditions there are always macrophages which are very ready to migrate and others are very unwilling to leave the tube". Our results have revealed that the data for normal macrophage migration display a quasi-rectangular distribution from 30 to 130 mg; higher and lower values are exceptionally rare. In view of the difficulty of evaluation it is recommended that control chamber growth lower than 3 mm^2 should be disregarded when estimating migration inhibition. The cause of the increased migration of macrophages is not known, but migration inhibition being proportional to the control values, it does not present any problem in evaluation.

The other difficulty of the method is the within animal variability of migration, as shown by the high values for SD. In addition, it is difficult to

trace the migration area particularly in the case of controls with growth of the "lacy fun type" [35]. This difficulty does not arise in the case of inhibited migration owing to the clumping of cells and the strict borderline of the migration area. To overcome these difficulties it is recommended to work with a high number of parallels and to read the inhibited macrophage migration before the control, in order to reduce the observer's error.

The applied *in vitro* PPD dose varies between 2.5 and 300 μg/ml according to the literature [7, 35]. In our laboratory the non-toxic dose of PPD was less than 15 μg/ml, but its value should be determined under different experimental conditions (and for every antigen).

In spite of the literary data suggesting a possible stimulating effect of lower than "non-toxic" antigen doses, PPD in such low doses failed to influence macrophage migration.

Finally, it may be stated that the evaluation of the macrophage migration test presents many difficulties for the investigator [6, 16, 31], but being aware of these problems the method is fairly reproducible and may be a useful tool for the *in vitro* measurement of delayed hypersensitivity.

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MACROPHAGE MIGRATION INHIBITION AS AN IN VITRO CORRELATE OF CUTANEOUS DELAYED SKIN REACTION

II. INVESTIGATIONS ON BCG VACCINATED GUINEA PIGS

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Summary. The connexion between skin testing, the most common method for the estimation of delayed type hypersensitivity, and the recently elaborated macrophage migration inhibition test has not been satisfactorily settled. The divergence of the two reactions has been established in experiments carried out in BCG vaccinated guinea pigs. Data are presented to show that a positive skin test is associated with a positive macrophage migration inhibition test but in the case of negative skin tests the macrophage migration inhibition test often shows positivity. The use of an elevated PPD dose for skin testing did not improve the correlation of the parameters investigated.

Delayed type skin reactions have been frequently used as diagnostic tests for tuberculosis or for the control of BCG vaccination and in several conditions accompanied by cellular type immunity [1, 2]. Several authors [3–5] have stressed the drawbacks of this test the function of which is not yet fully appreciated [6].

The recently elaborated macrophage migration inhibition (MMI) test seems to be a good measure of cellular immunity [7–10] possessing several common characteristics with the delayed type skin reactions [11, 12] and lacking any correlation with circulating antibodies. There are several observations on the divergence of the MMI test and the delayed type skin reactions [13–16], thus their interrelation does not seem to be settled satisfactorily.

The present paper reports on a study of the correlation of MMI and delayed type skin reactions in BCG vaccinated guinea pigs.

Materials and methods

Guinea pigs of both sexes weighing 250–300 g were used in the experiments. BCG vaccinated animals (10^4–10^5 viable units subcutaneously) were skin tested by 10 IU PPD (Purified Protein Derivative, Statens Serum Institute, Copenhange) at least 4 weeks after immunization and not more than 7 days prior to MMI testing. The largest diameter of the induration was measured to the nearest millimetre on the second day. In later experiments 10 and 100 IU PPD were simultaneously administered intracutaneously. The MMI method has been presented in part I of this paper [17]; the highest PPD doses non-toxic *in vitro* (10 and 15 μg/ml) were applied for MMI testing with the use of 6 parallels for the control and the antigen chambers.

Results

Fig. 1 presents data of MMI performed with 10 μg/ml PPD in relation to the diameter of PPD induced skin reaction in 76 BCG vaccinated guinea pigs. For the sake of brevity, data for MMI obtained by 15 μg/ml PPD are omitted, because their inhibitory activity though somewhat more expressed, was similar to that of the former dose (59 and 68% inhibition, respectively).

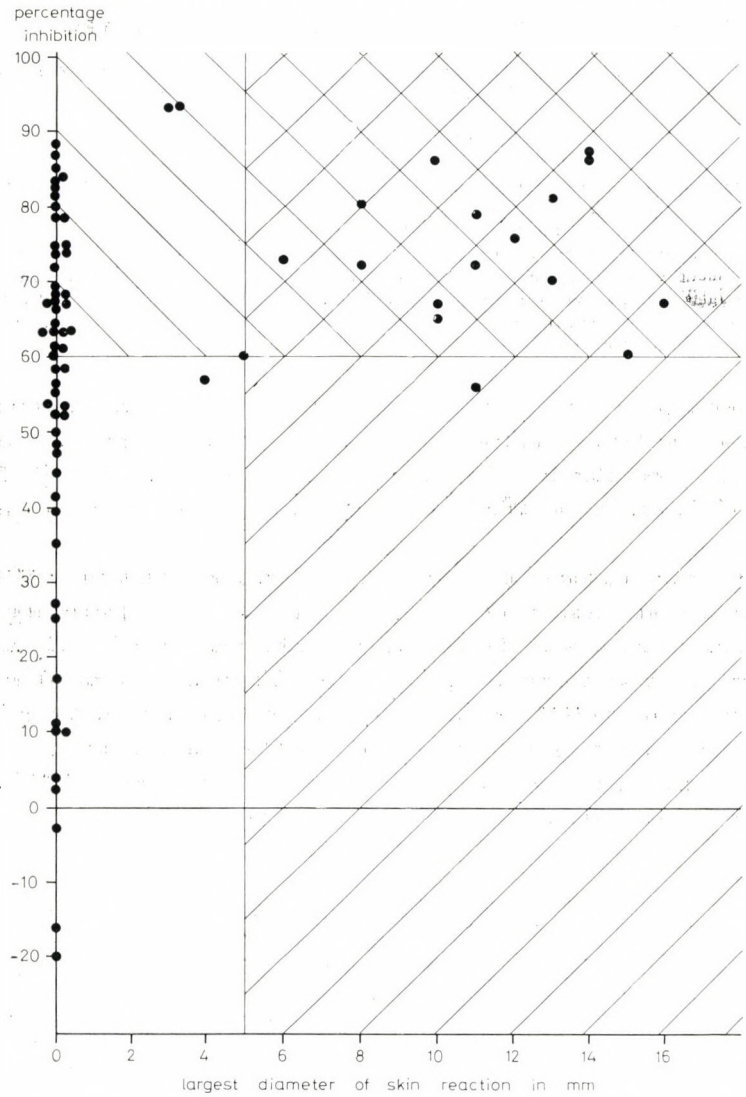

Fig. 1. Inhibition of migration of macrophages originating from BCG vaccinated guinea pigs in the presence of 10 μg/ml PPD, compared to skin reactions induced by intracutaneously administered 10 IU PPD (n = 76). Shaded area means positivity of migration inhibition and of skin reaction

The smallest skin reaction to 10 IU PPD was always accompanied by a considerably inhibited macrophage migration. But when the skin reaction is negative, the MMI is still positive in non-negligible numbers.

The possibility arose of a more close interrelation between skin tests and MMI in the case of higher intracutaneous PPD doses.

Fig. 2 demonstrates the data for MMI of 47 BCG vaccinated animals skin tested with 10 and 100 IU PPD. These animals did not respond to intracuta-

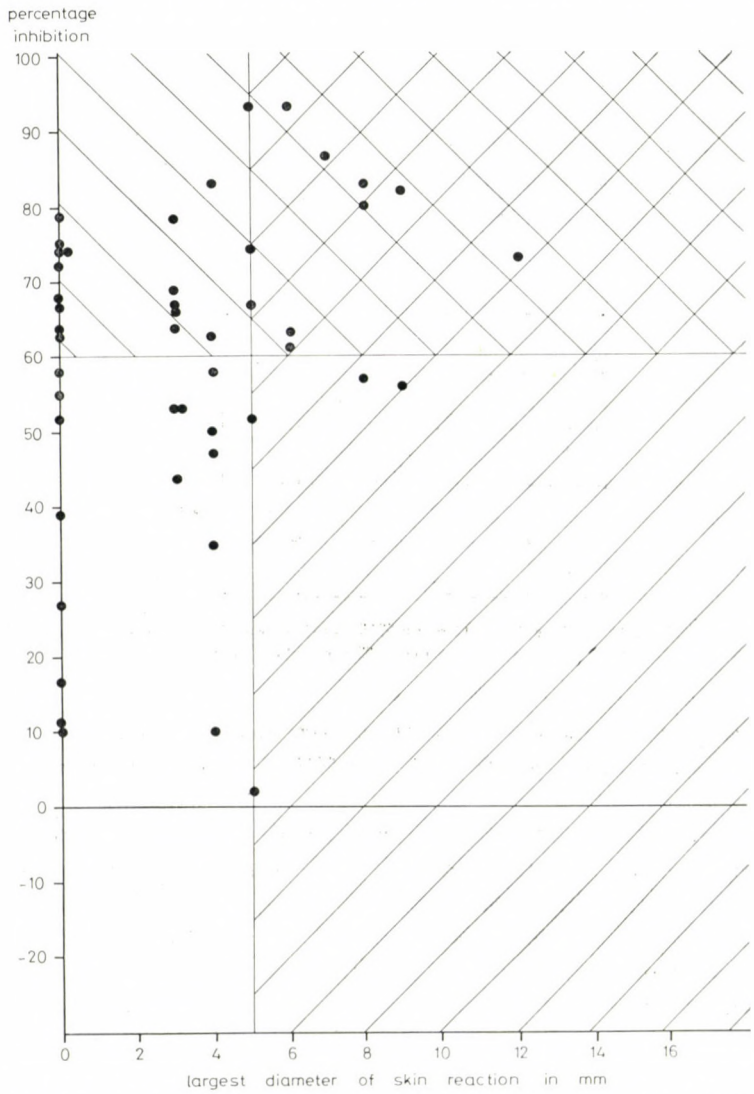

Fig. 2. Percentage inhibition of migration of macrophages originating from BCG vaccinated guinea pigs in the presence of 10 μg/ml PPD, compared to skin reactions induced by intracutaneously administered 100 IU PPD. The animals did not react to 10 IU PPD (n = 46). Shaded area means positivity of migration inhibition and of skin reaction

neously administered 10 IU PPD (they were selected from a triple number of simultaneously BCG vaccinated guinea pigs). No closer interrelation between the skin test and MMI could be found than in the former series. Moreover, sometimes a skin test positivity was associated with a negative MMI.

Part A

SR 100 u \ MMI 10 µg	+	±	−	Σ	
+	14	3	Ø	17	
±	2	1	Ø	3	
−	29	16	11	56	52 %
Σ	45	20	11	76	

Part B

SR 10 u \ MMI comm.	+	±	−	Σ	
+	17	Ø	Ø	17	
±	3	Ø	Ø	3	
−	37	8	11	56	66 %
Σ	57	8	11	76	

Fig. 3. Tabulated form of correlation between MMI and delayed type skin reaction due to 10 IU PPD i. c. administered. More details see in text

To complete the data, some animals were examined which had a positive skin test to 10 as well as to 100 PPD. The MMI was of course positive in these animals.

To establish the suggested divergence of the examined parameters of cellular immunity, the data were ranged into arbitrary groups of positive, negative and intermediate results.

In Fig. 3, the skin test was considered positive to 10 IU PPD when the largest diameter of the induration was 5 mm or more; negative when there was no reaction; 1–4 mm reactions were regarded as intermediate ones. The MMI was classified as positive when migration inhibition was more than 60%, and negative, when it was 30% or less. Between these two values, inhibition was considered intermediate. In part A, the evaluation of MMI based upon data obtained by the use of 10 µg/ml PPD *in vitro*; in part B, these data were based on common evaluation of MMI performed with 10 and 15 µg/ml PPD as follows.

The MMI was regarded as positive if at least one of the applied doses yielded a positive result; negative, if the MMI was negative with at least one of the PPD doses; and intermediate, if macrophage migration displayed an intermediate inhibition with both PPD doses. Contradictory results have not been observed.

Part A

SR 100 u \ MMI 10 µg	+	±	−	Σ
+	11	3	1	15
±	7	7	1	15
−	9	4	4	17
Σ	27	14	6	47

53 %

Part B

SR 100 u \ MMI comm	+	±	−	Σ
+	14	Ø	1	15
±	12	3	1	16
−	10	2	4	16
Σ	36	5	6	47

63 %

Fig. 4. **Tabulated form of correlation between MMI and delayed type skin reaction (SR) due to 100 IU PPD i.c. administered. More details see in text**

Fig. 3 shows that the positive skin tests were accompanied in most (part A) if not all (part B) of the cases by a positive MMI. Positive MMI reactions were also frequently met with in about 52 to 66% of negative skin reactions.

In Fig. 4 the same data are presented, with the only difference that the results obtained with intracutaneously administered 100 IU PPD are compared with those of the MMI. There was no apparent change in the frequency of negative skin tests associated with a positive MMI, but in some cases a negative MMI occurred with positive or intermediate skin reactions. Consequently, statistical analysis by means of the χ^2 test yielded a significant difference between the two reactions.

Discussion

It has been found that a negative skin test does not necessarily reflect a lack of immunological competence. Some patients with overwhelming tuberculous infection do not respond and the evaluation of the efficiency of BCG vaccination may be difficult in cases with a negative skin reaction [18].

According to the results, after BCG vaccination, the MMI test is more frequently positive than the skin test. If it could be proved that this difference does not yet mean a lower degree of specificity of the MMI test, then this *in vitro* reaction might be a useful tool in the establishment of cellular immune reactions.

Furthermore, MMI *in vitro* seems to have many advantages over the skin tests *in vivo*. First, a single mediator, the migration inhibitory factor (MIF) of known chemical properties may be considered responsible for the MMI, while in the delayed type skin reactions a great number of mediators may play a role [13, 19]. The quantitative or qualitative change of these substances may alter the manifestation of skin reaction even in the case of developed cellular immunity. Furthermore, the animal's general condition does not seem to influence the MMI while there may be several known and unknown factors altering the *in vivo* skin tests. The small antigen dose repeatedly administered into the skin may change the state of hypersensitivity (and may elicit unwanted effects) which is not the case with the MMI. Finally, the positivity of MMI seems to depend on the availability of a very few sensitized lymphocytes, probably T lymphocytes [20] (though data on MIF production by B lymphocytes may also be found [21]), whereas skin test positivity needs the presence of other mononuclear cells, too. This has been clearly shown by the experiments of VISACORPI [22] who sensitized X-irradiated (800 r) rats by bovine serum albumin in complete Freund's adjuvant and this resulted in the suppression of the skin reaction on the 0–5th post-irradiation day. At the same time the MMI was positive. Transfer of bone marrow cells was needed for restoring the skin reaction. From this, VISACORPI inferred "it seems that post-irradiation suppression of the skin reaction does not depend on damage of specific cells but depends on shortage of a bone marrow derived cell type participating in skin reactions as a non-specific component".

In spite of its drawbacks, the skin test is widely accepted for the examination of tuberculin sensitivity, due perhaps to its simple technique. Once its sufficient specificity has been established, the MMI may be a useful tool for the completion of data concerning the development of cellular immunity especially in the case of a negative skin test.

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STUDIES ON C4 INACTIVATOR INDUCED BY ADENOVIRUSES IN KB CELL CULTURES

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Summary. Infection of KB cell cultures with adenovirus prototype strains 3, 4, 11, and 13 induced an increase in the C4 inactivating activity of the medium while infection with virus types 1, 2, 5, 6, 7, 12, and 14 failed to cause any significant change as compared to the control. Adenovirus strains of the same types isolated from patients behaved as their respective prototype strains. A temperature resistant C4 inactivator of low molecular weight undetectable in the media of uninfected KB cell cultures was found to be responsible for the increase in C4 inactivating activity. No correlation could be found between the C4 inactivator inducing capacity of the strains studied and their ability to cause early CP changes.

The results of VON ZEIPEL's [1] and our previous studies have shown that KB cell cultures originating from a human mandibular carcinoma produce three substances different in molecular weight, exhibiting anti-complementary (AC) activity each of which inactivates human C4. Of these substances, the temperature sensitive one showing the highest molecular weight (above 200,000) is identical with C1 esterase, the activated first component of human complement [1–3]. As compared to the controls, infection with poliovirus type 1 resulted in a significant decrease while that with adenovirus type 3 led to a significant increase in the C4 inactivating activity of the media of KB cell cultures in 24 and 48 hr. Our earlier experiments using gel filtration through Sephadex G-100 columns indicated that the latter phenomenon might be attributed to the production of a temperature resistant C4 inactivating substance by cells infected with adenovirus type 3. No similar substance could be detected in the media of uninfected cells [2].

In the present work, the following problems have been studied. (i) Was the induction of the temperature resistant C4 inactivator (t.r. C4IA) brought about exclusively by adenovirus type 3 or was it a biological property characteristic of adenoviruses in general? (ii) Was there a correlation between the capacity of adenoviruses to induce t.r. C4 IA and their ability to cause early cytopathogenic (CP) changes? (iii) Finally, some properties of the t.r. C4IA produced in response to adenovirus type 3 infection have been studied.

Materials and methods

Viruses. Adenovirus prototype strains 1, 2, 3, 4, 5, 6, 7, 11, 12, 13 and 14, and adenovirus strains 1, 2, 3, 5, 6, 7 and 14 isolated in our laboratory were used.

Cell cultures and their infection. KB cells were grown in a medium containing 16% inactivated human serum, 3% of a 5% aqueous stock solution of lactalbumin hydrolysate, 19% Parker's medium 199, and 62% Hanks' BSS. Prior to infection, 6-day-old cultures were washed three times with Parker's medium 199 in order to remove human serum from the cultures. From each virus type, 0.1 ml undiluted virus suspension was used for infection. For maintenance, Parker's medium 199 alone was used.

Measurement of C4 inactivating activity. Cell cultures were frozen and thawed three times 48 and 72 hr after infection (*viz.* when complete CP effects have been attained) and then the media were harvested and centrifuged at 2500 rpm. The supernatants were incubated at 56 °C for 30 min and concentrated by freeze-drying to about one sixth of their original volume. The concentrated media were dialysed against veronal buffer (VBS, pH 7.4) overnight. C4 inactivating activity was then determined by the method of LAURELL and SIBOO [4].

Sephadex gel filtration. Four ml aliquots of the concentrated culture media of KB cells were put on Sephadex (Sephadex G-100 course, Pharmacia, Uppsala) columns measuring 260 mm in length and 20 mm in diameter. For elution, 90—100 ml VBS was used. A total of 28 3 ml fractions were collected. Elution speed was 15 ml/hr.

Trypsin sensitivity. After incubation of 0.5 ml aliquots of the fractions containing t.r. C4IA with 0.2 ml aliquots of a 5 μg/ml trypsin solution prepared of crystalline trypsin (Difco) at 37 °C for 2 hr, the C4 inactivating activity was determined immediately.

Estimation of molecular weight. A mixture of 3 substances of known molecular weight (Blue dextran 2×10^6, haemoglobin – 65 000 and K_2CrO_4–194) was run through the Sephadex columns used for the separation of the media of KB cell cultures. The molecular weight of t.r. C4IA was estimated from the V/V_0 values [5].

Immunodiffusion. Double diffusion was carried out in 1% agar (Difco) dissolved in VBS pH 8.6 containing 0.01% merthiolate. Holes 5 mm in diameter were located at 4 mm distance.

Complement fixation. Both cold (at 4 °C overnight) and heat (at 37 °C for 20 min) bindings were done in parallel by Takátsy's micro-method [6].

Results

1. Temperature resistant C4 inactivator inducing capacity of prototype adenovirus strains and strains isolated from patients. KB cell cultures were infected with prototype strains 1, 2, 3, 4, 5, 6, 7, 11, 12, 13, and 14. After incubation for 48 and 72 hr the media were harvested and heat-treated at 56 °C for 30 min. After concentration, the C4 inactivating activity of the media was determined. As controls, the media of uninfected KB cell cultures of the same passage number treated in the same way, were used. C4 inactivating activity was determined as the number of 50% haemolytic units (CH_{50}) bound from 10 CH_{50}. The fidelity of this method was $\pm 9.6\%$ in our studies and therefore only types fixing more than 1 of the 10 CH_{50}, *viz.* types 3, 4, 11 and 13, were considered positive as regards t.r. C4IA inducing capacity (Table I). In the media of the uninfected control cultures heat-treated at 56 °C for 30 min, no C4 inactivating activity could be detected.

The same experiments were performed also with adenoviruses 1, 2, 3, 5, 6, 7, and 14 isolated from patients. As shown in Table II, strains isolated from patients behaved exactly as the corresponding prototype strains: all

Table I

Temperature resistant C4 inactivator inducing capacity of adenovirus prototypes

Adenovirus type	C4 inactivating activity (number of CH_{50} bound from 10 CH_{50})
1	0.27
2	0.00
3	*1.44*
4	*2.12*
5	0.00
6	0.13
7	0.00
11	*1.44*
12	0.88
13	*1.95*
14	0.00

strains of the adenovirus type 3 studied inactivated more than 1 CH_{50}, while types 1, 2, 5, 6, 7, and 14 failed to exert any significant C4 inactivating effect.

2. Early CP factor and heat-resistant C4 inactivator inducing capacity of various prototype adenovirus strains. In these experiments, the early CP changes inducing and the C4 inactivating effects of the media heat-treated at 56 °C for 30 min of the KB cell cultures infected with the prototype strains studied in the previous experiments were investigated in parallel. KB cell cultures were checked after the inoculation of media both concentrated and non-concentrated for early CP effect (5 and 24 hr after inoculation) (Table III). It was only adenovirus type 11 which proved positive for both properties. Strains 1, 2, 5 and 6 resulted in early CP effects, but failed to induce the production of t.r.C4IA, while types 4 and 13 failed to induce CP effects even if concentrated, but they proved positive for C4 inactivator inducing capacity. Types 3 and 7 induced a CP effect only after concentration and the latter was negative also for the C4 inactivator inducing capacity.

3. Some properties of the temperature resistant C4 inactivator. In our earlier studies, the C4 inactivator substance induced by adenovirus type 3 was isolated on Sephadex G-100 columns, and we could show that its activity was not destroyed by heat-treatment at 56 °C for 30 min nor blocked by C1 esterase inhibitor [2].

In the present studies it has been found that isolated t.r.C4IA lost its activity when treated with trypsin at 5 μg/ml concentration at 37 °C for 2 hr. Trypsin at the applied concentration failed to influence the C4 titre of the human serum used for the titration of the C4 inactivating activity.

Table II

Temperature resistant C4 inactivator inducing capacity of adenovirus prototype strains and strains isolated from patients

Adenovirus type	Strain	C4 inactivating activity (number of CH_{50} bound from 10 CH_{50})
	Prototype	0.27
	1646/71	0.45
1	164/72	0.00
	1564/71	0.00
	346/72	0.00
	Prototype	0.00
	741/71	0.00
2	765/71	0.00
	613/71	0.00
	253/72	0.21
	Prototype	1.44
	344/71	1.21
3	695/71	1.07
	26/66	2.80
	406/72	2.26
5	Prototype	0.00
	144/72	0.22
	Prototype	0.13
6	1692/71	0.00
	1971/71	0.13
	633/72	0.00
	Prototype	0.00
	1206/71	0.03
7	1336/71	0.00
	1553/71	0.00
	357/71	0.00
	Prototype	0.00
14	1285/71	0.00
	83/68	0.16

Table III

Early CP factor and temperature resistant C4 inactivator inducing capacity of different adenovirus types

Adenovirus type	Early CP effect		C4 inactivating activity of the concentrated medium
	medium non-concentrated	medium concentrated	
1	+	+	−
2	+	+	−
3	−	±	+
4	−	−	+
5	+	+	−
6	+	+	−
7	−	±	−
11	+	+	+
12	−	−	±
13	−	−	+
14	.	.	−

. not tested

From Sephadex G-100 columns, t.r.C4IA eluted at 72–78 ml elution volumes, suggesting a molecular weight of 500–1000.

Using radial immunodiffusion and complement fixation test, an attempt was made to detect adenovirus antigen in fractions containing t.r.C4IA. As immune serum, a mixture of rabbit serum immunized against adenovirus type 3 and a pool of convalescent human sera was used. As controls, the fractions of the exclusion peak certainly containing adenovirus antigens and those of other peaks showing C4 inactivating activity were analyzed by both methods. Precipitation bands and specific complement fixation were obtained only with fractions of the exclusion peak while the fractions containing t.r.C4IA proved negative by both methods.

Discussion

Complement fixing virus antigens produced in tissue cultures often exert an AC effect [7, 8]. In our studies, infection of KB cell cultures with certain adenovirus types also resulted in an increase in the AC activity of the media. This phenomenon was due to the induction by the virus of a substance inactivating human C4 in the cell cultures. The substance is relatively temperature resistant and could not be detected in the media of uninfected cultures.

For studying the t.r.C4IA capacity of the different types of adenovirus, types from each of the four haemagglutination subgroups were selected. As

shown by the results, types 3, 4, 11, and 13 proved positive suggesting that the t.r.C4IA inducing property is not characteristic of either of the serological groups. This was supported by the finding that all virus strains isolated from patients behaved as the corresponding prototypes.

Adenoviruses are known to induce early CP changes in tissue cultures, 4–6 hr after infection. The cytotoxic factor responsible for the early CP effect shows high titres in some of the types, *viz.* types 1, 2, 5, 6, 11, 14 and 16, while it is hardly detectable *e.g.* in types 3, 4 and 7 [9–11]. In our experiments, the possible correlation between the t.r.C4IA inducing capacity and the early CP effect of the adenoviruses has been investigated. The assumption was based on that the cytotoxic factor – similarly to the t.r.C4IA– was relatively temperature resistant, could not be dialysed and was detectable only in the media of cultures infected with undiluted virus [9–13].

In agreement with data in the literature, early CP changes were observed to occur with types 1, 2, 5, 6 and 11. The fact that in the types studied the t.r.C4IA inducing capacity and the early CP effect may appear together or by themselves speaks against there being a correlation between these two biological properties.

The t.r.C4IA substance isolated from the media of KB cell cultures infected with adenovirus type 3 seems to be a polypeptide of low molecular weight. Trypsinization caused a loss of its activity. Its molecular weight is between 500 and 1000 as estimated by gel filtration, though the actual value may be somewhat higher since some polypeptides may be adsorbed to the gel and thus elute later [14]. Our experiments with polyacrylamide gel electrophoresis are still in progress; they seem to support the above data on molecular weight.

The fact that the C4 inactivator substance induced by adenovirus type 3 is definitely different from the early cytotoxic factor (*viz.* the free vertex capsomeres responsible for the early CP effect) [15, 16] and our failure to detect virus antigens in the fractions containing t.r.C4IA by either complement fixation or by immunodiffusion, support the assumption that t.r.C4IA is a new cellular product formed as a result of virus infection rather than a virion specific substance.

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RESISTANCE TO LISTERIA MONOCYTOGENES OF GUINEA PIGS SENSITIZED WITH A SIMPLE PROTEIN

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Summary. Guinea pigs injected with bovine albumin in complete Freund's adjuvant developed specific cutaneous sensitivity. Migration of their peritoneal macrophage was inhibited by the bovine albumin, and anti-bovine antibodies appeared in their sera. Four weeks after sensitization the animals were given another intraperitoneal albumin injection and were infected with *Listeria monocytogenes*. As a control, non-sensitized animals were challenged similarly. The resistance to infection was characterized by counting listeriae isolated from the spleen of infected animals. Twenty-four hours after infection there was no considerable difference in this respect between sensitized and control animals. Five days after challenge, however, the germ count was higher for the sensitized than for the control animals.

It is well-known that organisms infected by facultative intracellular parasites (*Mycobacterium*, *Listeria*, *Brucella*, *Salmonella*) develop delayed-type hypersensitivity (DTH) to the antigens of the infectious agent and a resistance to reinfection. The resistance can be transmitted passively with cells, but not with serum. For this reason, this type of resistance is termed cellular resistance (CR) [1]. The CR thus developed acts not only against the original pathogen but also against antigenically unrelated and even phylogenetically distant micro-organisms [2]. According to MACKANESS [3] DTH and antimicrobial CR are inseparable phenomena. The main factor of CR is the increased microbicidal capacity of the macrophages, which is attributable to soluble mediators released from sensitized lymphocytes on the antigenic stimulus. The manifestation of CR against microbes other than the primary pathogen is explained by the fact, that besides the macrophages originating in the sensitized organism, normal macrophages are also activated by the mediators. Among the experimental evidences supporting the MACKANESS theory those published by PATTERSON and YOUMANS are the most convincing [4]. These authors cultivated, with *M. tuberculosis*, lymphocytes from animals infected by the same bacterium. The cell-free supernatant of the culture inhibited the multiplication of virulent *M. tuberculosis* within normal marcophages in tissue culture.

While the causal relationship between infection allergy and the CR has universally been accepted, it is not clear whether a similar relationship existed between DTH of other origin and CR.

GOIHMAN-YAHR *et al.* [5] claimed that the microbicidal capacity of

guinea pig macrophages is not influenced by either contact dermatitis or rejection immunity. FORBES [6] found no increase in the resistance to *Listeria* of mice sensitized by ovalbumin. Others, however, have published positive results. DODD and NASH [7] sensitized and reinjected guinea pigs with bovine gamma globulin and subsequently challenged them with *Listeria*. One or two days after challenge the *Listeria* count was lower in the spleen of these animals than in the non-sensitized controls or in the sensitized animals that had not been reinjected with gamma globulin before listeria infection. SIMON and SHEAGREN [8, 9] incubated peritoneal exudate cells of guinea pigs sensitized by bovine gamma globulin or by picrylated human serum albumin with the corresponding antigen *in vitro*. Due to the specific antigenic stimulation the listericidal capacity of the peritoneal macrophages showed a considerable increase. Publications concerning the relationship between DTH of non-infectious origin and antimicrobial CR are full of contradictions, due perhaps to differences in the nature of the sensitizing antigens. Since, however, in the corresponding experiments the experimental models were always different, it seems impossible to estimate the role played by the quality of the antigen.

To eliminate the differences in the experimental model, we have adopted the schedule applied by DODD and NASH [7] in investigating the resistance to listeria infection of guinea pigs sensitized by a simple protein with the only difference that instead of bovine gamma globulin, we used bovine albumin as the sensitizing antigen.

Materials and methods

Experimental animals. Random-bred guinea pigs, 300—350 g in body weight, from the Institute's breed were used throughout.

Albumin. Bovine albumin Fraction V (Browning Chemical Corporation).

Adjuvant. Freund's complete adjuvant (Difco).

Listeria monocytogenes. Strain NCTC 7973 maintained on Dorset medium.

Experimental schedule for the examination of resistance to listeria infection of sensitized guinea pigs. Sensitization: 10 mg albumin in 1 ml isotonic NaCl solution were mixed with an equal volume of Freund's complete adjuvant. From the mixture 0.05 ml was injected into each of the four foot pads of each guinea pig.

Albumin reinjection and listeria infection. In the 4th week following sensitization the sensitized guinea pigs as well as an equal number of non-sensitized control animals of the same age and body weight were injected i.p. with 1 mg albumin dissolved in saline. Two hours later all the animals were infected with 10^8 germs obtained from a 48 hr culture on serum agar medium [10] of *Listeria*. One or 5 days after infection the animals were killed by a blow on the nape and their spleens were homogenized. The number of living *Listeria* cells were determined by culturing on blood agar plates at 37 °C for 48 hr. The germ count was calculated for the whole spleen.

Skin test. Hundred μg albumin dissolved in 0.2 ml saline was injected intradermally into the shaved skin of guinea pigs. The results were read 24 hr later.

Titration of anti-albumin antibody by passive haemagglutination (HA). Sensitized sheep erythrocytes were prepared as described by BOYDEN [11]. One ml 2% tanninized erythrocyte suspension was sensitized by 1 mg albumin. The reagent thus obtained was incubated in the presence of 10% formalin overnight at room temperature. Then the formalin was washed off and the brown cell sediment was resuspended in a phosphate-buffered saline of pH 7.4 containing 0.5% normal heat-inactivated rabbit serum and thiomersal at a dilution of 1 : 10,000.

Two per cent suspensions could be kept at 4 °C for 4—6 weeks without loss of sensitivity. As reference preparation, an anti-bovine albumin rabbit serum (Institute for Serobacteriological Production and Research Human, Budapest) was applied. Titration of this preparation was included in each experiment. As indicated by the constant titre of the reference preparation, the erythrocyte suspensions prepared at different times were equal in sensitivity. The guinea pig sera to be tested were inactivated at 56 °C for 30 min. Passive HA titration was performed by TAKÁTSY's micro-method [12].

Migration inhibition test. Guinea pigs were injected i.p. with 30 ml sterile paraffin oil and, 4 days later, killed by a blow on the nape. The peritoneal cells were washed off and subsequently washed three times with Hanks' solution. The last sediment was resuspended, depending on its quantity, in 0.3–0.4 ml Parker 199 solution containing 20% guinea pig serum with 100 U penicillin and 50 μg streptomycin per ml. The suspension thus obtained was distributed in glass capillary tubes 1.1 mm in diameter. The air-space end of the tubes was sealed over flame and the capillaries were centrifuged, and cut at the cell-fluid interphase. The migration chamber consisted of a 30 × 50 mm glass plate with a plastic ring 15 mm in diameter fixed to the plate by silicon wax. The upper edge of the ring was smeared with silicon wax. The piece of capillary containing the cells was fixed to the bottom of the chamber and this was immediately filled with the medium. The medium in the control chamber was the same as the suspending medium: that in the antigen chamber contained, in addition, 1 mg bovine albumin/ml. The cells obtained from each guinea pig were examined in 5 antigen chambers and 5 control chambers. The chambers, tightly covered by a cover slip, were incubated at 37 °C overnight. Then the migration areas were projected onto a sheet of paper at × 15 magnification, cut out and weighed on an analytical balance. Based on statistical analysis of the parallel values, inhibition was considered significant if the migration area in the antigen chamber was 70% or less of that in the control chamber.

Results

1. Development of sensitivity to albumin (Table I). Four days after the sensitizing injection the DTH skin reaction of some animals already had a

Table I

Dynamics of specific skin sensitivity of guinea pigs sensitized with bovine albumin

Days after sensitization	No. of animals tested	Distribution of skin reactions by maximum diameter, mm			
		<5	5–10	11–20	21+
2	8	8	—	—	—
4	8	1	4	3	—
7	8	—	1	7	—
28	9	—	1	2	6

diameter of 5 mm or more. By the 7th day all the animals showed a positive skin test. On the 28th day necrotic lesions with uneven outlines were frequent. It should be noted that the animals were used on a sole occasion, so the reactions were not influenced by previous intracutaneous tests.

2. Dynamics of the migration tests. In Fig. 1 prints represent individual migration inhibition values. Migration inhibition was expressed by the following index

$$\text{Mi} = \frac{\text{migration area in antigen chamber}}{\text{migration area in control chamber}} \times 100$$

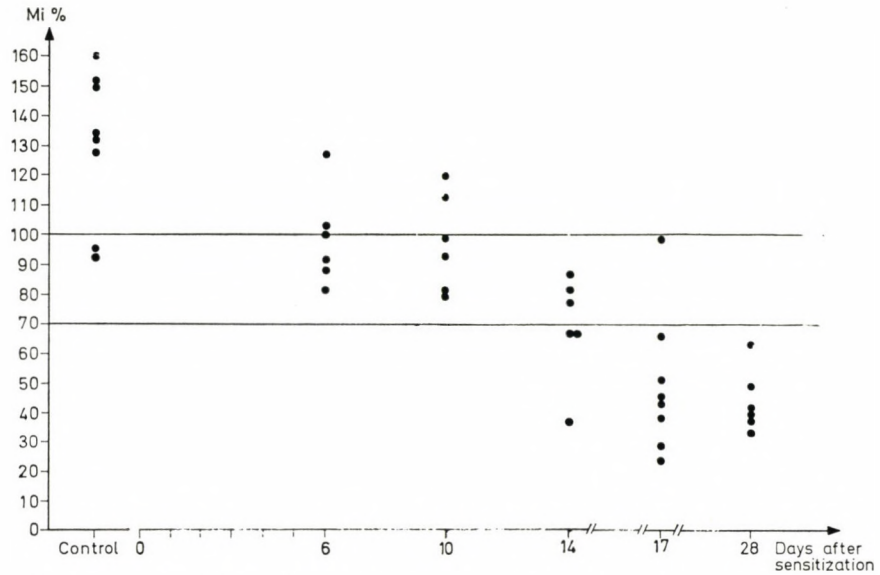

Fig. 1. Antigen-induced inhibition of the migration of exudate cells of guinea pigs sensitized with bovine albumin

It is clear that 1 mg/ml albumin in the medium instead of inhibiting even stimulated migration of the cells of normal animals. Cells from guinea pigs treated with albumin-adjuvant 6 or 10 days previously did not show extremely high (150–160%) values. In half of the cases the migration value did not even reach 100%. Thus, in the presence of antigen there was a perceptible, but statistically not significant, difference in cell migration between sensitized and normal animals. Statistically significant inhibition first appeared on the 14th day, and by the 28th day all the sensitized animals proved positive.

3. Dynamics of anti-albumin antibodies (Table II). Specific serum antibodies were demonstrated in 7 of the 10 animals as soon as on the 7th day post injection.

Table II

Dynamics of passive HA serum antibodies in guinea pigs sensitized with bovine albumin

Days after sensitization	No. of animals tested	Distribution of sera by titre*									
		<4	4	8	16	32	64–512	1024	2048	4096	8192
4	8	8	—	—	—	—	—	—	—	—	—
7	10	3	2	4	—	1	—	—	—	—	—
28	8	—	—	—	1	1	—	3	2	—	1

* Reciprocals

4. Listeria level in the spleen of sensitized and non-sensitized guinea pigs was determined 1 and 5 days after albumin challenge and listeria infection in four repeated experiments each. In Fig. 2 data of repeated tests have been combined.

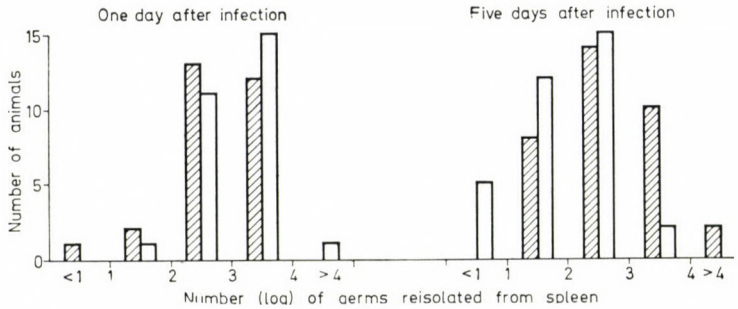

Fig. 2. Distribution of albumin-sensitized and non-sensitized guinea pigs by listeria count in the spleen, 1 and 5 days after infection; shaded columns = sensitized, clear columns = untreated

Twenty-four hours after infection there was no appreciable difference between the sensitized and control groups, except a tendency towards lower values in the sensitized group. However, 5 days after infection, there was a striking difference between the two groups, but surprisingly in the opposite direction. Higher values occurred more frequently in the sensitized than in the control group.

Discussion

The observation that 24 hr after listeria infection the sensitized animals did not appear to be more resistant than the non-sensitized ones is consistent with the data published by GOIHMAN-YAHR *et al.* [5]. Accordingly, CR is not influenced by DTH of non-infectious origin. Considering that we adopted the model of DODD and NASH [7] and these authors observed a correlation between DTH and CR, the lack of correlation in our experiments might be attributed to a difference in the sensitizing antigens. In the literature bovine albumin is considered to be of poor antigenicity for the guinea pig. In our experiments, however, typical DTH skin reactions could be elicited by intracutaneously applied albumin as soon as 4 to 7 days after the sensitizing injection; furthermore, on the 28th day, (*i.e.*, at the time of the listeria infection, in challenge experiments) albumin invariably inhibited the migration of peritoneal cells of sensitized animals. Thus we have succeeded in inducing DTH in guinea pigs with bovine albumin administered in Freund's adjuvant. On the other hand, the titres of the passive HA test clearly show that the sensitization resulted in a mixed type hypersensitivity. Although corresponding

data have not been published by DODD and NASH [7], there is hardly any doubt that specific antibodies can be induced by bovine gamma globulin, too, if given in Freund's complete adjuvant. Consequently, the divergence of the results should not be attributed to differences in the antibody status. It seems reasonable to assume that the DTH induced by albumin, unlike that induced by gamma globulin, is not accompanied by a resistance to intracellular infection. This assumption cannot, however, be accepted unless the guinea pigs sensitized with bovine gamma globulin would show an increased resistance to listeria infection under our experimental conditions as well. Such experiments are in progress.

The increased listeria counts in the spleen of our sensitized animals on the fifth day following infection was surprising. To our best knowledge, similar observations have not been published. We tend to attribute this phenomenon to the relatively large challenge dose of albumin given before the infection. With regard to the results published by JOKIPII and JOKIPII [13], it may be assumed that the sensitized lymphocytes were desensitized rather than stimulated by the albumin challenge. An experimental evidence for this would support the connection between DTH and CR from a new side, on the basis of the decreased resistance manifesting itself on the 5th day after infection.

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COLONIZATION OF SPECIFIC R^+ ENTEROBACTERIACEAE IN THE INTESTINES OF HOSPITALIZED NEWBORNS AND BABIES

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Summary. About 90% of 128 hospitalized newborns and babies acquired independently of antibiotic therapy *Escherichia coli* and *Enterobacter cloacae* strains bearing *fer(f)*$_{II}$ *chl str sul mer tet amp* and *fer(f)*$_{III}$ *chl str sul mer amp kan(neo)* plasmids, very often as hetero-R-types. These R^+ bacteria could be demonstrated to be present throughout the hospital.

The spread of drug resistance (R-plasmids) among *Enterobacteriaceae* is due to their ability of conjugative transmission and a selection of R^+ bacteria by antibiotics applied for therapeutical and feeding purposes [1–5].

The incidence of R-plasmid bearing bacteria increases from year to year. Differences in their frequency depend upon the species investigated and the number of R^+ bacteria in the individual countries [1–3, 6–15].

Observations concerning the incidence of R plasmids are rather instructive in view of R-plasmid investigations in non-pathogenic bacteria [1, 8, 11]. These species are thought to be the reservoir of R-plasmids [1, 2, 3, 6, 15]. Such investigations have recently demonstrated that about 60% of healthy children and adults are excreting enteric bacteria with transferable single tetracycline resistance and less than 10% with transferable multiple drug resistance [16]. The growing incidence of transferable tetracycline resistance among such bacteria coincides very probably with the selection pressure in favour of tetracycline resistant bacteria by tetracycline feeding in animal farms and the assumption of a direct or indirect transfer of R-plasmids from animal to human bacterial strains [3–5].

This environmental antibiotic influence on the selection of drug resistant bacteria probably occurs also under hospital conditions. However, several authors have failed in finding a significant difference in the prevalence of R^+ bacteria in hospitalized subjects treated with antibiotics [6, 7, 14, 17, 18].

To gain detailed information concerning this question, the faecal flora of hospitalized newborns and babies treated with and without antibiotics has been investigated with regard to the incidence and types of R-plasmids.

Materials and methods

Bacteria. The following *Escherichia coli* K-12 strains were used in the experiments: VT 452 *(arg⁻ met⁻ leu⁻ his⁻ str-r nal-r)*, VT 2093 *(thi⁻ thr⁻ leu⁻ trp⁻ his⁻ str-r nal-r)*, Hfr(H) *(thi⁻)* and the K-12 wild type. All strains were obtained from the culture collection of the Institute of Experimental Epidemiology.

Phages. Determination of the sex-pilli of R⁺Hfr and R⁺K-12 strains was carried out with the F-specific bacteriophage fr (5.0×10^9). The restriction capacity (hsp-locus) of the plasmids investigated was determined by the use of bacteriophage (1.5×10^9). All bacteriophages were obtained from the culture collection of the Institute of Experimental Epidemiology.

Media. All media used were described previously [19].

Conjugation techniques and the experiments to characterize the plasmids genetically were also described previously [19, 20].

Isolation and determination of the bacteria. Faecal samples of the newborns and babies investigated were streaked directly on agar-plates containing tetracycline (20 μg/ml) and chloramphenicol (20 μg/ml) and on Endo-agar. The colonies were characterized according to TSCHÄPE *et al.* [16].

Air samples. Air samples were obtained with settle plates with an exposure time of 1 hr. The media of the settle plates were the same as those used for the isolation of bacteria from faecal samples. The colonies were characterized according to TSCHÄPE *et al.* [16].

Results

Isolation and characterization of R-plasmids. During their stay in hospital, 128 newborns and babies were observed in respect to *Enterobacteriaceae* with R-plasmids.

Of 628 examined samples, 572 yielded multiple drug resistant (91.0%) and 38 single (tetracycline) drug resistant (6.0%) bacteria. In 18 samples sensitive or other bacteria could be found (Table I).

Table I

Drug-resistant Enterobacteriaceae isolated from newborns and babies

No. of infants	No. of samples	Total incidence	
		Multiple	Single
128	628	572 (91.0%)	38 (6.0%)

For transferable R-plasmids, 230 multiple and single drug resistant bacteria were studied; 91.5% of the single resistant bacteria and 89.0% of the multiple resistant bacteria exhibited transferable R-plasmids. The frequency of transmission was between 10^{-3} and 10^{-5} per recipient cell (Table II).

The transferred resistant determinants (Table II) and the *fer*-properties of more than 100 isolated R-plasmids (Table III) were characterized. The overwhelming majority of the plasmids analyzed was of the type $fer(f)_{II}$ *chl str sul*

mer tet amp (58.5%) and of the type *fer(f)*$_{III}$ *chl str sul mer amp kan(neo)* (32.0%). Table III represents some selected R-plasmids. Of all strains, 18.5% contained hetero-R-types indicating that both plasmids belonged to different compatibility sets. As demonstrated in Table IV, *fer(f)*$_{II}$ was classified with the same compatibility groups as R222 [15], whereas *fer(f)*$_{III}$ plasmids did not reveal superfiction immunity against *fer(f)*$_{II}$ or *fer(i)* plasmids.

Table II

Transmission of resistance

No. of strains	Resistance type	Transmission	Transferred properties
200	multiple drug resistance	89.0%	*chl str sul mer tet amp* *chl str sul mer amp kan (neo)* *str sul mer tet amp*
30	single drug resistance (tetracycline)	91.0%	*tet*

These two R-plasmids behaved as linked structures. Segregants from the individual plasmids could not be isolated. The *fer(f)*$_{II}$ and *fer(f)*$_{III}$ plasmids did not reveal any restriction capacity to bacteriophage.

Epidemiological observations. The occurrence of a limited number of plasmid types (Table V) was a remarkable feature. This was underlined by the fact that during the first days of life, about 90% of the hospitalized newborns had acquired *E. coli* and *Enterobacter cloacae* strains (Table VI) bearing the analysed plasmids *fer(f)*$_{II}$ *chl str sul mer tet amp* and *fer(f)*$_{III}$ *chl str sul mer amp kan(neo)* (often as hetero-R-types). These results are summarized in Fig. 1.

Moreover, infants treated with antibiotics acquired, independently of the antibiotic used, also *E. coli* and *E. cloacae* strains with *fer(f)*$_{II}$ *chl str sul mer tet amp* and *fer(f)*$_{III}$ *chl str sul mer tet amp kan(neo)* plasmids whether or not *Enterobacteriaceae* with other plasmid types were present (Table VII).

Colonization of a limited number of bacterial strains carrying the two mentioned types of R-plasmids led to the assumption that these bacteria exist in the hospital environment or in food (*e.g.* milk). The mothers' milk fed to the babies was not contaminated with R+ *Enterobacteriaceae.*

In order to examine the hospital environment for airborne bacteria with the specific plasmids isolated from the babies' faeces, air samples were obtained by means of settle plates.

After 1 hr exposure, expecially *E. coli* and *E. cloacae* strains bearing the plasmids *fer(f)*$_{II}$ *chl str sul mer tet amp* and *fer(f)*$_{III}$ *chl str sul mer amp kan(neo)* were found. These results are summarized in Tables V and VI. They demon-

Table III

Genetic characterization of R-plasmids isolated from bacteria of infants

Designation	Properties	fr. lysis[4]		Fertility inhibition		Frequency[6] of transmission	fer-type
		R+Hfr	R+K12	his[5]	trp[5]		
RH223[1]	*tet*	—	—	1.0	1.0	10^{-3}	fer(f)
RH248	*chl str sul mer tet amp*	—	—	0.3	1.0	10^{-3}	fer(f)
RH256	*chl str sul mer tet amp*	—	—	3.5	0.8	10^{-4}	fer(f)
RH286	*chl str sul mer amp kan (neo)*	—	—	0.7	0.7	10^{-5}	fer(f)
RH506	*chl str sul mer tet amp*	—	—	1.0	0.2	10^{-3}	fer(f)
RH517	*chl str sul mer amp kan (neo)*	—	—	5.0	1.5	10^{-5}	fer(f)
RH523	*tet*	—	—	6.5	3.0	10^{-3}	fer(f)
RH652	*chl str sul mer tet amp*	—	—	11.0	0.1	10^{-3}	fer(f)
RH809-1[2]	*chl str sul mer tet amp*	—	—	7.0	3.5	10^{-4}	fer(f)
RH809-2[2]	*chl str sul mer amp kan (neo)*	—	—	0.4	1.0	10^{-4}	fer(f)
RH897	*chl str sul mer tet amp*	—	—	0.1	0.1	10^{-4}	fer(f)
RH1076	*tet*	ol	—	91.0	99.0	10^{-4}	fer(i)
RH1077	*tet*	—	—	2.0	4.5	10^{-5}	fer(f)
RH1085	*tet*	—	—	3.5	0.8	10^{-3}	fer(f)
RH1236	*chl str sul mer tet amp*	—	—	3.5	2.0	10^{-5}	fer(f)
RH IV[3]	*chl str sul mer amp kan (neo)*	—	—	1.5	2.5	10^{-4}	fer(f)
RH XV	*tet*	ol	—	93.5	92.0	10^{-3}	fer(i)
RH XVIII	*chl str sul mer tet amp*	—	—	5.5	6.0	10^{-3}	fer(f)
R222	*chl str sul mer tet*	—	—	7.0	2.0	10^{-4}	fer(f)
R^-	—	ol	—	100.0	100.0	—	—

[1] R-plasmids isolated from the faecal flora of hospitalized infants
[2] Both types of R-plasmid isolated from one clone (hetero-R-type)
[3] R-plasmids isolated from air-born bacteria in the hospital
[4] Phage fr is used at a concentration of 3.5×10^9 p.f.u./ml; ol = opaque lysis
[5] The figures show the frequency of prototrophic colonies of R^+ Hfr $\times$ VT 2093 in per cent, as compared to the control (= 100%) R^- Hfr$\times$VT 2039
[6] Frequency of transmission calculated according to the activity of conjugation of a 14-day-old agar slant R^+ culture

strate the incidence of *fer(f)*$_{II}$ *chl str sul mer tet amp* or *fer(f)*$_{III}$ *chl str sul mer amp kan(neo)* in *E. coli* and *E. cloacae* from the faeces of newborns and babies as well as from the hospital environment. It is noteworthy that – even during a period of 50 ward days – a few children (4 in 128) did not acquire the *E. coli* and *E. cloacae* strains bearing the mentioned R-plasmids.

Table IV

Superinfection data for different RH-plasmids in isogenic E. coli K12 strains and R222

Donors VT451	Recipients (CSH-2)					
	R⁻	517	809–2	809-1	XVIII	222
RH517	100[2]	—	—	99.0	78.0	75.0
RH809-2[1]	100	—	—	98.0	93.0	90.0
RH809-1[1]	100	98.0	100	—	—	0.1
RH XVIII	100	95.0	83.5	—	—	0.1
R222	100	68.5	89.0	0.1	0.1	—

[1] RH809-1 and RH809-2 were isolated from one cell (hetero-R)
[2] The figures show the percentual frequency of transmission

Properties of the plasmids:
RH517 = *fer(f)$_{III}$ chl str sul mer amp kan (neo)*
RH809-2 = *fer(f)$_{III}$ chl str sul mer amp kan (neo)*
RH809-1 = *fer(f)$_{II}$ chl str sul mer tet amp*
RHXVIII = *fer(f)$_{II}$ chl str sul mer tet amp*
R222 (W) = *fer(f)$_{II}$ chl str sul mer tet*

Table V

Frequency of R-plasmids isolated from Enterobacteriaceae of hospitalized newborns and babies and of air samples in the ward

R-plasmid type	Faeces, per cent	Air samples, per cent
fer(f) chl str sul mer tet amp	58.5	45.0
fer(f) str sul mer tet amp	3.5	—
fer(f) chl str sul mer amp kan (neo)	32.0	16.0
fer(f) tet	2.0	19.0
fer(i) tet	4.0	20.0

Table VI

Frequency of bacterial species isolated from newborns and babies and from air samples of the ward

Bacterial species	Faeces, per cent	Air samples, per cent
Escherichia coli	53.0	60.0
Enterobacter cloacae	22.5	26.5
Proteus mirabilis	13.0	—
Proteus morganii	0.5	—
Klebsiella aerogenes	8.0	7.5
Citrobacter freundii	2.0	—
Pseudomonas aeruginosa	1.0	6.0

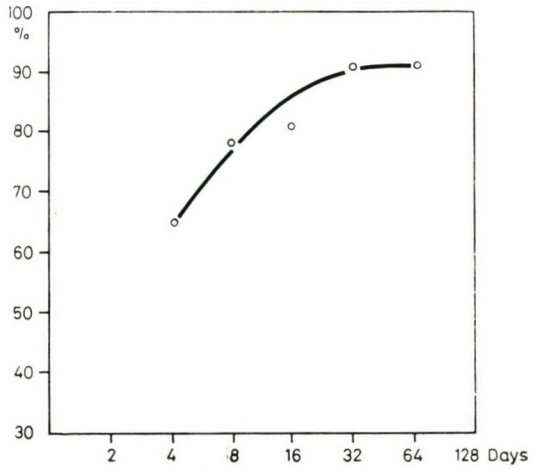

Fig. 1. Colonization in the intestines of 85 newborns of distinct multiple drug resistant bacteria during 4 up to 64 ward days. R^+ bacteria with *fer(f)*$_{II}$ *chl str sul mer tet amp or fer(f)*$_{III}$ *chl str sul mer amp kan(neo)* (See text)

Table VII

Incidence of multiple drug resistant bacteria in the faeces of infants before and after oral antibiotic therapy

Plasmid type	No. of babies before antibiotic therapy	No. of babies after antibiotic therapy
fer(f) chl str sul mer tet amp	5[1]	23
fer(f) chl str sul mer amp kan (neo)	—	5
fer(i)/fer(f) tet	12	1
no plasmids	13	1

[1] Infants with *Enterobacteriaceae* carrying the plasmid *fer(f) chl str sul met tet amp* had been hospitalized several days before the first sample was taken

Discussion

In the faecal flora of 128 newborns and babies, multiple drug resistant bacteria were demonstrated in 91% and single tetracycline resistant bacteria in not more than 6% of the 628 samples investigated. Of the multiple drug resistant bacteria 89% and of the single tetracycline resistant bacteria 91% were due to transferable R-plasmids. The R-plasmids isolated from *E. coli* and *E. cloacae* were mainly of two types: *fer(f)*$_{II}$ *chl str sul mer tet amp* (58%) and *fer(f)*$_{III}$ *chl sul mer amp kan(neo)* (32%), which often occurred as hetero-R-types. Observation of a limited number of bacterial species bearing the two

types of transferable plasmids was underlined by the fact that newborns had preferably acquired the bacterial strains with the specific plasmids mentioned. Moreover, babies subjected to antibiotic therapy acquired independently of the antibiotics used *E. coli* and *E. cloacae* strains with the *fer(f)*$_{II}$ *chl str sul mer tet amp* or the *fer(f)*$_{III}$ *chl str sul mer amp kan(neo)* plasmid whether or not *Enterobacteriaceae* with other plasmid types had been present before hospitalization.

It has been shown by means of the settle-plate method that the same bacteria with the same R-plasmids had to be present in the hospital. In the present study, the hospital environment rather than food or milk was the source of contamination. Mother's milk fed to the babies was not contaminated with coliform R+ bacteria and could therefore be excluded as a potential source of infection.

The existence of bacteria spreading over the hospital in spite of strict hygienic measures is a well-known fact [6, 9, 18, 21, 23]. In the last years, interest has especially been focussed upon pathogenic bacteria and the development of antibiotic resistance among them. This has led to a neglect of the appearance of bacteria which are just beginning their development in pathologic processes and of the prevalence of multiple drug resistance (R-plasmids) among the so-called non-pathogenic bacteria as potential reservoirs of R-plasmids. This has been described as an environmental pollution due to drug resistance [9].

Apathogenic bacteria may, however, acquire virulence under hospital circumstances (*e.g.* in stress situations) or by conjugative acquisition of virulence-plasmids [cf. 22]. This has been stressed in a case of grave meningitis caused by hospital strains (*E. cloacae*) carrying the *fer(f)*$_{II}$ *chl str sur mer tet amp* plasmids (to be published).

The incidence of R-plasmids, the bacterial strains isolated (*E. coli*, *E. cloacae*, *Klebsiella aerogenes*), and especially the types of R-plasmids isolated from hospital environment and from the faeces of the hospitalized newborns and babies (91%) were in contrast to the incidence and types of R-plasmids in bacteria from the intestines of healthy infants [16]. Among more than 1000 examined healthy infants, 55% exhibited *Enterobacteriaceae* bearing *fer(i) tet* or *fer(f) tet* plasmids, often linked with the *colI* determinant. Of the bacteria, 7.5% were infected with *fer(f) chl str sul mer tet* plasmids. Only 0.5—1% of the healthy infants was shown to excrete *E. coli* strains carrying R-plasmids similar to the types isolated from the hospital. The difference in the incidence of transferable single tetracycline resistance and multiple resistance among *Enterobacteriaceae* of healthy infants was found to be due to tetracycline feeding of animals. In the GDR, solely tetracyclines are used for this purpose.

Moreover, 90–100% of the workers employed in antibiotic (tetracycline) plants, in the food industry applying tetracycline additives or in

animal production with tetracycline feeding have been shown to harbour *Enterobacteriaceae* with transferable tetracycline resistance (to be published).

Obviously, such a selection pressure exists also under hospital conditions, but different of the type of the drug resistance selected. The selection of transferable multiple drug resistance in non-pathogenic bacteria in the hospitals explains that other authors have found no significant differences between hospitalized patients with and without antibiotic therapy [7, 18, 23].

The results presented in this paper point to the problem of environmental pollution by drug resistance and are calling for a careful antibiotic policy not only in animal feeding but also in the hospitals.

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CULTURAL AND BIOCHEMICAL PROPERTIES OF ACHOLEPLASMA AND MYCOPLASMA STRAINS OF CATTLE ORIGIN

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Summary. Cultivation and biochemical properties of microorganisms isolated from cattle and classified with the order *Mycoplasmatales*, have been studied. The strains studied corresponded to the criteria of classification in the order *Mycoplasmatales*. On the basis of their cholesterol requirement, digitonin and sodium-polyanethol-sulphonate sensitivity, not only the *Acholeplasma laidlawii* A PG8 species but also LEACH's group 6 (PG49) and AL-AUBAIDI's K (B107 PA) strain were found to belong to the *Acholeplasmataceae* and the others to the *Mycoplasmataceae* family. The galactose, xylose, esculin and arbutin fermentation tests as well as the determination of carotenoid synthesis provide suitable means for the differentiation of *Acholeplasma* species. Determination of glucose, arginine and sorbitol fermentation, phosphatase activity, film and spot formation as well as of the serum digesting capacity, serves for the preliminary definition of *Mycoplasma* species.

The classification of microorganisms within the order *Mycoplasmatales* is based on the absence of cell wall, typical colony morphology, filterability through 450 nm membrane filter and lack of reversion under appropriate conditions. On the basis of their cholesterol requirement [1] the organisms of the order *Mycoplasmatales* belong to the families *Mycoplasmataceae* or *Acholeplasmataceae* which include one genus each. To the first family belongs the *Mycoplasma* genus which includes the T strains too [2]; to the second family belongs the genus *Acholeplasma*. In the *Mycoplasma* genus 33 species and in the *Acholeplasma* genus 3 species are distinguished [3, 4].

The purpose of the present studies was to select suitable culturing methods for bovine mycoplasmas and acholeplasmas other than T strains and to compare their characteristics. Interest was focussed on differentiation of the individual species with special regard to the criteria of classification into the order *Mycoplasmatales*, *viz.* the families *Mycoplasmataceae* and *Acholeplasmataceae*.

Materials and methods

Strains. A total of 17 strains was included in the studies (Table I). Of these, 7 strains were representatives of a *Mycoplasma* or *Acholeplasma* species or subspecies, 3 strains (PG49, PG50, and PG51) represented LEACH's [5] serogroups 6, 7 and 8, and 4 strains (B129P, B142P, B107PA and B144P) the H, I, K and L serogroups of AL-AUBAIDI and FABRICANT [6], while strain M165/69 belonged to the group isolated by LANGFORD and DORWARD [7] from epidemic keratoconjunctivitis of cattle. The strains *M. agalactiae* subsp. *agalactiae* PG2 of sheep origin, and *M. mycoides* subsp. *capri* PG3 of the goat were included in the studies because they resem-

Table I

Mycoplasma type strains and reference strains of cattle origin

Species or serogroup	Strain			
	Type or reference	Origin	Isolation	Origin (organ)
M. dispar	462/2	NCTC	GOURLAY (1970)	cattle lung
M. bovirhinis	PG43 (5M 331)	NCTC	HARBOURNE *et al.* (1965)	cattle lung
M. mycoides subsp. *mycoides*	PG1	E. A. FREUNDT (D. G. ff. EDWARD)	LAIDLAW (1931)	cattle lung
M. mycoides subsp. *capri*	PG3	E. A. FREUNDT (D. G. ff. EDWARD)	CHU (1954)	goat lung
AL-AUBAIDI group L	B144 P	J. FABRICANT	MOULTON (1956)	cattle joints
LEACH group 7	PG50(N29)	D. G. ff. EDWARD	SIMMONS and JOHNSTON (1963)	cattle joints
M. bovigenitalium	PG11(B2)	E. A. FREUNDT (D. G. ff. EDWARD)	EDWARD (1950)	cattle genitals
M. agalactiae subsp. *agalactiae*	PG2	E. A. FREUNDT (D. G. ff. EDWARD)	LOPEZ (1934)	sheep udder
M. agalactiae subsp. *bovis*	Donetta	D. G. ff. EDWARD	HALE *et al.* (1962)	cattle udder
M. arginini	G230	M. F. BARILE	MORRIS (1968)	mouse brain
LEACH group 8	PG51 (M47/67)	D. G. ff. EDWARD	HUDSON and ETHERIDGE (1963)	cattle nose
AL-AUBAIDI group H	B139 P	J. FABRICANT	AL-AUBAIDI (1970)	cattle uterus
AL-AUBAIDI group I	B142 P	J. FABRICANT	FABRICANT (1959)	cattle lung
Untyped	M165/69	NCTC	LANGFORD and DORWARD	cattle eye
A. laidlawii	PG8	E. A. FREUNDT	LAIDLAW and ELFORD (1936)	sewage-water
LEACH group 6	PG49 (Squire)	D. G. ff. EDWARD	LANGER and CARMICHAEL (1963)	cattle lung
AL-AUBAIDI group K	B/107 PA	J. FABRICANT	AL-AUBAIDI (1970)	?

NCTC = National Collection of Type Cultures, Colidale Av., London, N. W. 9.

bled in certain properties the bovine strains *M. agalactiae* subsp. *bovis* (Donetta) and *M. mycoides* subsp. *mycoides* PG1, respectively.

Culturing. On the basis of a 5-year experience, two standard media, B and N were chosen. Medium B has been described earlier [8]. Medium N contained Bacto brain heart infusion (Difco), 3.7 g; yeast extract (Difco), 0.5 g; distilled water, 100 ml; horse serum, 20 ml; 25% yeast extract [9], 10 ml; 10% thallium acetate, 1 ml; penicillin (200,000 U/ml), 1.3 ml; 0.2% deoxyribonucleic acid (Sigma), 1.3 ml; 50% glucose, 2.0 ml; pH 7.8.

To prepare a solid medium, agar was added before autoclave treatment. To study *M. dispar* strains, GOURLAY and LEACH's [10] as well as FRIIS' [11] media were used.

Optimum gaseous environment and temperature as well as the growth curves have been determined as described earlier [8].

Filtration experiments. Fourty-eight-hour-old broth cultures were passed through filters of 450 nm, (Gelman Metrical GA-6), 200 nm (Gelman Metrical GA-8), 100 nm (Millipore type VM) and 50 nm (Millipore type VM) pore size. Colony forming units (c.f.u.) were determined before and after filtration.

Morphology. Strains were cultured on agar at optimum temperature and atmosphere, and the colonies were studied under the stereomicroscope daily. In the log phase, the broth cultures were studied with dark field microscopy.

Reversibility. The strains were maintained on the above-described media, in the absence of penicillin and thallium acetate, through 5 passages.

Classification within Mycoplasmataceae and Acholeplasmataceae was based on cholesterol [12] requirement as well as on digitonin [8] and sodium polyanethol-sulphonate sensitivity.

Biochemical examinations. Arginine and urea decomposing activity [8], phosphatase activity [14], film and spot formation [15], serum digestion [14], triphenyl-tetrazolium chloride (TTC) reduction [16], sensitivity to erythromycin, polymyxin B and kanamycin [8], as well as haemolysis, haemagglutination and haemadsorption caused by the strains [17] have been studied. Modified medium B was used in these experiments except in the case of *M. dispar* and *Mycoplasma* 165/69 strains, for which FRIIS' and GOURLAY and LEACH's media were supplemented with the substrates mentioned and inoculated with 0.1 ml of the washed and 20-fold concentrated suspension of the strains.

Polyacrylamide-gel electrophoresis. Concentrated and washed mycoplasma suspensions were dissolved in a 2 : 1 : 0.5 mixture of phenol, acetic acid and water, and run in polyacrylamide containing 5 M urea and 35% acetic acid [18].

Carotenoid synthesis. These experiments were carried out with the technique of RAZIN and CLEVERDON [19].

Results

1. Culturing conditions

(a) *Culture media.* Except for *M. dispar* (462/2), all the strains showed satisfactory growth (10^7 c.f.u./ml) on medium B. Similar good results or slightly less favourable ones were observed on medium N. In view of its simple composition, medium B was used more frequently. Good results were obtained with *M. dispar* in FRIIS' fluid medium. Growth was less satisfactory on the same solid medium than on GOURLAY and LEACH's medium, on which 'fried egg' colonies were frequent.

(b) *Growth in different gaseous environments.* Fifteen strains grew equally well in the 4 different atmospheres. Strain M165/69 showed a considerably better growth in the presence of CO_2. A flask containing a burning candle provided the condition optimum for the growth of 462/2 *M. dispar* strain, which failed to grow under aerobic conditions.

(c) *Growth at different temperatures.* As Table II shows, the majority of strains failed to grow at 22 °C. *M. arginini* G230 grew slowly at this temperature and yielded a 5 exponents lower c.f.u. The same phenomenon was observed with strain PG50 belonging to LEACH's group 7. *M. mycoides* subsp. *capri* PG3 and strain PG 49 (which belongs to LEACH's group 6) yielded an identical c.f.u. at low temperatures and 37 °C, the growth rate being about 50% slower in the former case. On the other hand, *A. laidlawii* A PG8 and AL-AUBAIDI K(B107 PA) strains showed nearly identical c.f.u. and growth rate at both temperatures.

Table II

Growth of bovine Mycoplasma and Acholeplasma strains at different temperatures

Species or serogroup	Type or reference strain	Maximum c.f.u. and growth index					
		37 °C		27 °C		22 °C	
		c.f.u.	Index	c.f.u.	Index	c.f.u.	Index
M. dispar	462/2	10^5	3.6	3×10^4	1.8	0	0
M. bovirhinis	PG43	5×10^4	5.0	0	0	0	0
M. mycoides subsp. *mycoides*	PG1	5×10^7	7.5	6×10^2	0.2	0	0
M. mycoides subsp. *capri*	PG3	2×10^9	10.0	10^9	9.2	10^9	4.6
AL-AUBAIDI group L	B 144 P	3×10^8	9.0	3×10^8	4.6	0	0
LEACH group 7	PG50	10^9	10.0	10^9	8.0	10^4	0.8
M. bovigenitalium	PG11	5×10^7	7.6	$3+10^2$	0.2	0	0
M. agalactiae subsp. *agalactiae*	PG2	10^9	10.0	10^9	4.0	0	0
M. agalactiae subsp. *bovis*	Donetta	10^8	7.2	10^5	1.4	0	0
M. arginini	G230	3×10^7	8.0	10^7	5.4	10^2	1.0
LEACH group 8	PG51	3×10^8	9.0	0	0	0	0
AL-AUBAIDI group H	B 139 P	5×10^6	7.0	7×10^2	0.2	0	0
AL-AUBAIDI group I	B 142 P	10^8	9.0	6×10^7	5.2	0	0
Untyped	M165/69	10^7	5.4	2×10^5	3.8	0	0
A. laidlawii	PG8	2×10^8	9.0	2×10^8	7.8	10^8	8
LEACH group 6	PG49	5×10^5	5.8	5×10^5	5.2	2×10^5	3.8
AL-AUBAIDI group K	B 107 PA	3×10^8	9.0	2×10^8	8.4	2×10^8	7. 4

(d) *Growth curves.* Growth curves of the individual strains differed in rate, c.f.u./ml yield and survival time. Their majority reached the highest titre between the second and fourth day; acholeplasmas grew quicker than mycoplasmas. The M.165/69 and especially the *M. dispar* strain grew slowly. Significant differences were found in the decrease of c.f.u. count. A rapid decrease of c.f.u. occurred with *M. arginini* G230 two days after having reached the peak titre, while the decrease was essentially slower with *M. agalactiae* subsp. *bovis* (Donetta) of LEACH's group 8 (PG51). Under optimum conditions, all the strains reached a titre of at least 10^7 c.f.u./ml.

2. Filtration.

The smallest reproductive unit was larger than 100 nm and smaller than 200 nm with each of the strains studied.

3. Morphology

Typical 'fried egg' colonies were formed by each strain. This observation applied also to *M. dispar* though it formed prevalently atypical colonies. The size of the colonies varied between 0.25 mm (*M. dispar*) and 3 mm (*M. mycoides* subsp. *capri*). Under the dark-field microscope the strains showed a typical pleomorphous pattern, in which coccoid, filamentous and branching filamentous structures were visible. Buds arising from the filaments could be observed with some strains.

4. Reversibility

By the end of the fifth passage, all the strains formed typical fried egg colonies even on antibiotic- and thallium-free media, consequently they were not L-forms.

5. Classification within the Mycoplasmataceae and Acholeplasmataceae families

(a) *Cholesterol requirement.* Neither the *A. laidlawii* strains nor the AL-AUBAIDI's group K (B107 PA) and LEACH's group 6 strains (PG49) required cholesterol. Determination of the cholesterol requirement was unsuccessful with the *M. dispar* strain, as it failed to grow on the test media used. The strains AL-AUBAIDI H/B139P, I/B142P, L/144P, the M165/69 as well as LEACH's group 7 (PG50) and group 8 (PG51) and 6 nameless strains all required cholesterol (Table III).

Table III

Cholesterol requirement, digitonin and sodium-polyanethol-sulphonate sensitivity of bovine Mycoplasma and Acheloplasma strains

Species or serogroup	Type or reference strain	Cholesterol requirement	Digitonin sensitivity, mm	Sodium-polyanethol-sulphonate sensitivity, mm
M. dispar	462/2	not examined	12.0	7.5
M. bovirhinis	PG43	+	4.5	3.0
M. mycoides subsp. *mycoides*	PG1	+	8.0	9.5
M. mycoides subsp. *capri*	PG3	+	3.0	3.5
AL-AUBAIDI group L	B 144 P	+	6.0	5.0
LEACH group 7	PG50	+	4.5	4.5
M. bovigenitalium	PG11	+	10.0	6.0
M. agalactiae subsp. *agalactiae*	PG2	+	7.0	7.0
M. agalactiae subsp. *bovis*	Donetta	+	7.0	6.0
M. arginini	G230	+	7.0	2.0
LEACH group 8	PG51	+	5.0	5.0
AL-AUBAIDI group H	B 139 P	+	7.0	1.0
AL-AUBAIDI group I	B 142 P	+	3.0	0.0
Untyped	M165/69	+	10.0	5.0
A. laidlawii	PG8	0	0.0	0.0
LEACH group 6	PG49	0	0.0	0.0
AL-AUBAIDI group K	B 107 PA	0	0.0	0.0

(b) *Digitonin and sodium polyanethol sulphonate sensitivity. A. laidlawii* A PG8, LEACH's group 6 (PG49) and the AL-AUBAIDI K (B107 PA) strains were resistant to digitonin, while the inhibition zone was 3 to 12 mm wide with the remaining strains. Four strains, three of the digitonin resistant ones and AL-AUBAIDI I/B142P were resistant to sodium polyanethol sulphonate. With the other strains, the inhibition zone was 1.0 to 7.5 mm wide (Table III).

6. Biochemical studies

The strains studied were all negative in the urea, sucrose and mannitol tests. For the differentiation of digitonin resistant strains which do not require cholesterol, *i.e.* acholeplasmas, the galactose, xylose, arbutin and aesculin fermentation tests are suitable (Table IV).

Table IV

Differentiation of bovine Acholeplasma species

Species or group	Reference strain	Galactose	Xylose	Aesculin	Arbutin	Carotenoid	Polymyxin B
A. laidlawii A	PG8	−	−	+	−	+	R
LEACH group 6	PG 49 Squire	−	−	−	−	−	S
AL-AUBAIDI group K	B107 PA	+	+	+	+	−	R

S = sensitive, R = resistant

At the same time all these strains gave a positive glucose test under both aerobic and anaerobic conditions, positive cellobiose and TTC reduction tests, negative mannose, sorbitol, arginine, phosphatase reactions, and did not show film and spot formation and serum digestion. All the strains gave beta-haemolysis with guinea pig erythrocytes, alpha and in some cases beta-haemolysis with bovine and sheep erythrocytes. They agglutinated guinea pig erythrocytes, but not cattle and sheep erythrocytes. None of the strains showed haemadsorption with guinea pig and cattle erythrocytes. The following tests appeared to be necessary for further differentiation of the cholesterol-requiring, digitonin-sensitive strains of the genus *Mycoplasma*: glucose and arginine fermentation, phosphatase activity, film and spot formation and serum digesting capacity. The first two tests allowed to divide the *Mycoplasma* genus into 3 groups.

(a) *M. mycoides* subsp. *mycoides*, *M. mycoides* subsp. *capri*, the AL-AUBAIDI L-group, LEACH's group 7. *M. dispar* and *M. bovirhinis* belonged to the glucose-positive and arginine-negative group, which was characterized by mannose fermentation, TTC reduction, alpha and beta-haemolysis with guinea

pig, cattle and sheep erythrocytes, high erythromycin sensitivity, by the lack of cellobiose, galactose, xylose, aesculin and arbutin fermentation as well as a lack of haemadsorption. Glucose oxidation, phosphatase activity, film and spot formation, serum digestive and sorbitol fermentation properties, kanamycin and polymyxin B sensitivity provided means for a further differentiation within this group (Table V).

(b) *M. arginini*, LEACH's group 8, H and I groups of AL-AUBAIDI and the untyped M165/69 strains belonged to the arginine-positive and glucose-negative group. The latter strain was found to form acid; however, as this did result from glucose fermentation, the determination of arginine fermentation was rather difficult.

The strains in this group were uniformly negative in mannose, galactose, cellobiose, xylose, sorbitol, aesculin, arbutin-splitting and serum digestion tests; they failed to agglutinate cattle erythrocytes, but showed alpha and sometimes beta-haemolysis with the erythrocytes tested, and were sensitive to kanamycin. For further differentiation, phosphatase activity, film and spot formation, TTC reduction, haemadsorption of guinea pig and cattle erythrocytes, agglutination of guinea pig erythrocytes as well as polymyxin B and erythromycin sensitivity tests were needed (Table VI).

(c) Three species coud be included in the glucose-negative and arginine-negative group: *M. bovigenitalium*, *M. agalactiae* subsp. *agalactiae* and the *M. agalactiae* subsp. *bovis* (Donetta) strain. They were all negative in the carbohydrate tests, none of them agglutinated cattle erythrocytes but all showed alpha or beta-haemolysis with the erythrocytes studied. All the three strains produced phosphatase, formed film and spot, and reduced TTC. However, they displayed differences in cattle erythrocyte adsorption, guinea pig erythrocyte haemagglutination and polymyxin B sensitivity (Table VII).

7. *Polyacrylamide-gel electrophoresis*

Each strain displayed a different behaviour. The electrophoretic analysis of certain separate species revealed significant differences. In the case of the strains *M. mycoides* subsp. *mycoides*, *M. mycoides* subsp. *capri*, and *M. agalactiae* subsp. *bovis* the differences did not suffice for stating that the strains studied belonged to separate species.

8. *Carotenoid synthesis*

Only strain *A. laidlawii* A PG8 was positive. The carotenoid synthetizing capacity of LEACH's group 6 and AL-AUBAIDI's K group could not be proved (Table I).

Table V

Biological properties of glucose-positive and arginine-negative Mycoplasma species

Species or serogroup	Reference strain	Glucose oxidation	Sorbitol decomposition	Serum digestion	Phosphatase activity	Film and spot formation	Guinea pig erythrocyte agglutination	Kanamycin	Polymyxin B
M. mycoides subsp. *mycoides*	PG1	+	−	−	−	−	+	S	R
M. bovirhinis	PG43	+	−	−	(+)	+(14 days)	+	S	S
M. mycoides subsp. *capri*	PG3	+	+	+	−	−	+	R	R
LEACH group 7	PG50	+	−	+	−	−	+	S	R
AL-AUBAIDI group L	B144P	+	−	+	−	−	+	S	R
M. dispar	462/2	−	−	−	−	−	−	S	S

S = sensitive, R = resistant

Table VI

Biological properties of arginine-positive and glucose-negative Mycoplasma species

Species or serogroup	Reference strain	Phosphatase activity	Film and spot formation	TTC reduction	Guinea pig erythrocyte adsorption	Cattle erythrocyte adsorption	Guinea pig erythrocyte agglutination	Polymyxin B	Erythromycin
M. arginini	G230	−	−	−	−	−	−	R	R
LEACH group 8	PG51	+	−	−	−	−	+	R	R
AL-AUBAIDI group H	B139P	−	−	−	−	−	−	S	S
AL-AUBAIDI group I	B142P	−	+	+	−	−	+	R	S
Untyped	M165/69	+	+ (14 days)	+	+	+	−	S	S

S = sensitive, R = resistant

Table VII

Biological properties of glucose-negative and arginine-negative Mycoplasma species

Species or group	Reference strain	Guinea pig erythrocyte adsorption	Cattle erythrocyte adsorption	Guinea pig erythrocyte agglutination	Polymyxin B
M. bovigenitalium	PG11	—	—	—	S
M. agalactiae subsp. *agalactiae*	PG2	+	+	—	S
M. agalactiae subsp. *bovis*	Donetta	—	—	+	R

S = sensitive, R = resistant

Discussion

The majority of bovine *Mycoplasma* and *Acholeplasma* species were successfully grown on standard media B and N. The same media seemed suitable for the first isolation of these species. Growth of *M. dispar* required, however, FRIIS' and GOURLAY and LEACH's medium. The majority of strains did not display essential differences in respect of the gaseous environment. At the same time, characteristic requirements must be taken into consideration with certain species such as *Mycoplasma* 165/69 and *M. dispar*.

As to the incubation temperature, acholeplasmas were found to be capable of growing at 22 °C. This property might be taken into account at classification. This criterion, however, is not an absolute one, as certain *Mycoplasma* species may also grow at low temperatures. Before detailed serological examination, it is advisable to determine the growth curve, to be able to perform the transfer in due time.

The morphology, filtrability, penicillin resistance and lack of reversion of the strains studied, excluded the possibility of their being L-forms.

The first step in the identification of isolates is their classification with the families *Mycoplasmataceae* or *Acheloplasmataceae*. Here is the strain's cholesterol requirement the criterion. For this purpose EDWARD's method [12] appeared suitable, but the less complicated sodium polyanethol sulphonate test recommended by KUNZE [13] is also adequate and the digitonin sensitivity test even more so. The last method is advisable with strains difficult to grow *e.g.* with *M. dispar*, since they do not grow on serum fraction and cholesterol-containing media.

In the present study, 3 *Acholeplasma* strains were demonstrated. The statement of EDWARD [12] has been confirmed concerning LEACH's group 6 strain (PG49). As to the AL-AUBAIDI K (B107 PA) strain [6], the authors have mentioned that similarly to the strain AL-AUBAIDI I (B142P), this one

could also be passaged on media devoid of serum and thus assumed that both were acholeplasmas. In our studies, only the AL-AUBAIDI K (B107 PA) strain was, however, found to be *Acholeplasma*. The AL-AUBAIDI I(B142P) strain was proved to require cholesterol, so we classified it with the family *Mycoplasmataceae*.

A further differentiation of cattle *Acholeplasma* is made on the basis of the galactose, xylose, aesculin and arbutin tests and carotenoid determination. The significance of the arbutin test must be emphasized. In other experiments we have shown that of acholeplasmas, only *A. axanthum* ferments arbutin. As this test was positive in the AL-AUBAIDI K group too, it appears necessary to investigate the relationship between *A. axanthum* and that group. This was indicated also by the lack of carotenoid synthesis, since carotenoid synthesis characterizes *A. laidlawii* and *A. granularum* but not *A. axanthum* [4]. Determination of glucose and arginine fermentation appears to be suitable for the grouping of species belonging to the *Mycoplasma* genus.

Determination of film and spot formation, phosphatase activity and serum-digestive capacity provides means for the further differentiation of species belonging to the individual groups. Our observation on the serum-digesting capacity of the AL-AUBAIDI L and LEACH's group 7 strains, which characterizes also certain *M. mycoides* strains, is emphasized. According to ALUOTTO *et al.* [14], *M. bovirhinis* may also digest serum, but our data failed to confirm this. Biochemical examination of *M. dispar* and *Mycoplasma* 165/69 is difficult. The former grows only on GOURLAY and LEACH's and FRIIS' medium. This fact in itself is sufficient for a preliminary diagnosis, which may further be confirmed by an examination in specific serum.

The results of antibiotic resistance tests differed in many respects from the data of AL-AUBAIDI and FABRICANT [6]. Our findings concerning haemadsorption were also different. In our experiments, *M. bovirhinis* failed to adsorb the erythrocytes, while all *M. bovirhinis* strains isolated in Czechoslovakia [20] displayed haemadsorbing capacity. The role of these tests in species determination is difficult to define; it appears to be necessary to examine several strains belonging to the same species or serogroup.

The use of reference sera is absolutely necessary for the final determination of the species. These tests are required for preliminary diagnosis, to reduce the necessary number of reference sera, and for the confirmation of the final diagnosis.

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VIRULENCE AND ACID AGGLUTINATION OF SHIGELLA

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Summary. A total of 710 *Shigella flexneri* and 290 *Shigella sonnei* strains were examined. Saline suspensions of virulent 24 hr cultures showed agglutination in one or more tubes of a buffer solution series in the range of pH 1.6—4.9. The majority of avirulent S-form cultures agglutinated in the range of pH 1.0—1.4 or not at all. *S. sonnei* phase II and some *S. flexneri* cultures reacted in all tubes at pH 1.4—4.9. Ninety per cent of *S. flexneri* and 75% of *S. sonnei* mixed phase cultures behaved in this manner. Pure phase I avirulent *S. sonnei* cultures were inagglutinable. Strains giving unusual types of acid agglutination consisted mostly of different colony variants: virulent and avirulent forms and, in *S. sonnei*, a variable ratio of phase I and phase II cells. The alteration of acid agglutination on heating at 100 °C for 2 hr, on washing in chloroform, 70% ethanol, acetone or 2% cetylpyridinium bromide was characteristic of the virulence of the organism. Acid agglutination was influenced by a change in the medium, namely when aqueous agar (10^{-3}–10^{-8} g/ml), albumin (10^{-3}–10^{-7} g/ml) or peptone (10^{-2}–10^{-5} g/ml) solutions were substituted for physiological saline.

The guinea pig eye reaction introduced by SERÉNY [1] is a reliable model for estimating the virulence of shigellae. In working with a great number of strains, the advantage of an *in vitro* method is obvious. In this paper an account is given of experiments with acid agglutination, a technique the reliability of which has been debated [2, 3].

Materials and methods

Reagents. A series of buffer solutions consisting of 15 tubes was prepared as shown in Table I [4—6].

Bacterial strains were freshly isolated from patients or symptomless carriers. Virulent and avirulent derivatives resulting from spontaneous variation or obtained by passaging on culture medium or after UV irradiation and strains stored at 5 °C were also tested.

Bacterial suspensions. The growth from 24 hr meat extract agar plates was suspended and, in part of the experiments, washed in 0.8% saline and adjusted to a density of 10^8–10^9 cells/ml. In some experiments the bacteria were resuspended in the supernatant of a multiple amount of culture centrifuged previously. Studies were also performed with 0.1% agar, 0.1% albumin, 0.1% peptone (Richter) and their 10^{-1}–10^{-5} dilutions as resuspending agents. Acid agglutination was examined after heating at 100 °C for 2 hr, and after washing in 70% ethanol, acetone, chloroform or 2% cetylpyridinium bromide.

Acid agglutination was carried out in 15×160 mm tubes. To each of 15 tubes 0.5 ml of the different buffer solutions, to one tube 0.5 ml saline was pipetted, then 1 ml bacterial suspension was added to each tube. Readings were made after 24 and 48 hr incubation at room temperature. The agglutination of virulent suspensions was finely granular and disintegrated readily on shaking. Therefore, ring formation was considered as positive reaction (+). Negative (—) tubes were characterized by a deposit of bacteria at the bottom. Inhomogeneous cultures

Table I

Preparation of buffer series for acid agglutination

	pH							
	1.0	1.2	1.3	1.4	1.6	1.9	2.3	2.6
0.1 N HCl, ml	300	270	240	210	180	150	120	90
0.1 M glycine, ml	—	30	60	90	120	150	180	210

	pH						
	2.9	3.3	3.7	4.0	4.3	4.6	4.9
0.1 N HCl, ml	60	30	15	132	107.1	69.3	13.2
0.1 M glycine, ml	240	270	285	—	—	—	—
0.1 M sodium citrate, ml	—	—	—	168	192.9	230.7	286.8

showed both ring formation and deposit, and the result was recorded as +— (ring formation predominant) and as —+ (deposit predominant). After shaking the tubes, the bacteria descended in a manner similar to the original reaction. *S. sonnei* phase II and *S. flexneri* R suspensions agglutinated in coarser grains and disintegrated less easily on shaking.

Passages were carried out on agar plates, in broth and in synthetic media containing different amino acids or $NH_4H_2PO_4$ as nitrogen source [7, 8].

Colony morphology was examined with a hand lens at reflected and transmitted light [9].

UV irradiation was performed at 250 nm and 20 cm distance for ½ to 16 min in a thin layer of 2 ½ hr peptone water culture.

Results

Results for *S. flexneri* are presented in Tables II and III. Suspensions prepared without and with washing in 0.8% saline gave similar patterns of acid agglutination. The majority of strains virulent by the guinea pig eye test reacted positively, while cultures not causing keratoconjunctivitis usually failed to agglutinate. Agglutination of unwashed bacteria began usually at more acid reactions (pH 1.0–1.2) than that of washed suspensions (pH 1.9).

As the pH range of agglutination varied considerably, the cultures were grouped in the following manner. For Table II (unwashed suspensions) the strains were grouped according to the upper pH limit of agglutination, since the lower limit was usually uniform (pH 1.0). For Table III (washed bacteria), in view of the variable lower limit, the following classification was made: group 3.3–3.7 contained strains showing agglutination in at least one tube within this range, although many of them reacted also outside of this range, down to pH 1.6 and up to pH 4.9; the rest of the groups was formed as in Table II, indicating the upper limit of agglutination. Bacteria giving agglutination up to pH 1.6 were partly virulent, partly avirulent; this type of reaction

Table II

Acid agglutination of unwashed S. flexneri cultures

	Acid agglutination positive, pH						Negative, pH	Total
	1.4	1.6	1.9–2.6	2.9	3.7	4.9	1.0–4.9	
Guinea pig eye test positive No. of strains	5	3	4	9	195	47	21	284
Guinea pig eye test negative, No. of strains	21	3	—	—	8	2	71	105

Table III

Acid agglutination of S. flexneri cultures after washing in saline

	Acid agglutination positive, pH					Negative, pH	Total
	1.6	1.9–2.6	2.9	3.3–3.7	1.0–4.9	1.0–4.9	
Guinea pig eye test positive, No. of strains	3	2	12	174	1	27	219
Guinea pig eye test negative, No. of strains	4	—	—	6	2	96	108

(shown by 2% of the strains) was unsuitable for the estimation of virulence. Three washed cultures agglutinated in every tube (pH 1.0–4.9), similarly to *S. sonnei* phase II strains which will be dealt with in a subsequent part of this paper. From Tables II and III it is evident that, if only 3 buffer solutions (pH 2.9, 3.3 and 3.7) are considered, unwashed and washed bacteria gave practically identical results.

Repeated experiments with atypically behaving 34 washed strains showed that the type of acid agglutination became congruent with virulence for 14 suspensions, while for 9 strains the result of the eye reaction changed so as to correspond to the acid agglutination pattern. The difference in the results of the first and the repeated experiments was attributable to an inhomogeneity or to a considerably decreased virulence of the cultures, as indicated by the following model experiments.

Virulent (acid-agglutinable) and avirulent (acid-inagglutinable) variants of the same strain were mixed at different proportions. In the presence of 20% avirulent cells only a partial (+—) agglutination, with 50% avirulent cells no agglutination was observed. At the same time a loopful of agar culture (approx. 10^9 cells) containing not more that an estimated amount of 1% virulent bacteria gave rise to keratoconjunctivitis. From the conjunctival discharge, virulent bacteria giving typical acid agglutination were isolated.

Table IV

Relationship of colony morphology with acid agglutination and virulence

Colony morphology	Result of acid agglutination as compared to guinea pig eye test			
	Congruent		Incongruent	
	Eye test positive	Eye test negative	Eye test positive	Eye test negative
G, No. of colonies	33	12	2	—
R, No. of colonies	11	42	2	1
N, No. of colonies	7	7	6	—

G = greenish glistening, R = reddish and N = net-structure colonies selected at oblique light

An alteration of the virulence of *S. flexneri* is frequently accompanied by the appearance of colony variants [10]. The relationship of colony variants with acid agglutination and virulence is shown in Table IV, where the behaviour of three kinds of colony variants of the same strains is demonstrated. Greenish (G) and net-structure (N) colonies were virulent in about 70%, while reddish (R) colonies in about 20%. With colonies G and R there was a good correlation between virulence and acid agglutination; about a third of colonies N gave discrepant results. Old, avirulent laboratory strains showed variable colony morphology and, with some exceptions, failed to agglutinate.

Virulent *S. sonnei* strains were usually inhomogeneous. On agar plates the serologically and morphologically distinct avirulent form appeared regularly in the first subculture [9]. The avirulent forms are stable: one of them corresponds to phase II, the other still contains phase I antigen and produces at transmitted light optically homogeneous 'S' colonies similar to those of *S. flexneri*. Acid agglutination of virulent *S. sonnei* was considerably influenced by the presence of phase II (Tables V and VI). Virulent cultures inhomogeneous in colony morphology mostly agglutinate over a wider pH range than *S. flexneri* and the type of agglutination is similar to that of *S. sonnei* II. Avirulent, morphologically homogeneous phase II cultures reacted over a wide pH range, while phase I S-form colonies, similarly to avirulent homogeneous *S. flexneri* cultures, failed to agglutinate. Examinations with washed cultures resulted in a sharper differentiation of virulent and avirulent strains.

The above examinations were performed with variants derived spontaneously upon serial passage or prolonged storage. After subjecting broth cultures to UV irradiation, the acid agglutination of agar plate subcultures was incongruent with the virulence test when both virulent and avirulent variants were present. After selecting pure cultures, no discrepancy was revealed.

Table V

Acid agglutination of unwashed S. sonnei cultures

pH spectrum	Agglutination	Guinea pig eye reaction positive, No. of *S. sonnei* I and II cultures	Guinea pig eye reaction negative, No. of *S. sonnei* II cultures
1.0–4.9 and 0.8% NaCl	+	—	10
1.0–4.9	+	8	18
1.0–3.7	+	58	10
1.2–4.9	+	33	—
1.0–4.9	—	15	—
Total		114	38

Table VI

Acid agglutination of S. sonnei cultures after washing in saline

pH spectrum	Agglutination	Guinea pig eye reaction positive, No. of *S. sonnei* I and II cultures	Guinea pig eye reaction negative, No. of *S. sonnei* cultures	
			I	II
1.0–4.9 and 0.8% NaCl	+	—	—	10
1.0–4.9	+	1	—	13
1.2–4.6	+	83	—	—
1.0–4.9	—	20	12	—
Total		104	12	23

The aim of subsequent experiments was to decrease the number of discrepant results. Washing in saline of agar cultures resulted in a narrowing of the range of acid agglutination, especially at low pH values. If the washed bacteria were resuspended either in their own original supernatant or in the supernatant of the culture of virulent or avirulent shigellae or of other bacteria, the range of acid agglutination became similar to that exhibited by unwashed bacteria. Dilution of the supernatant resulted in a gradual narrowing of the agglutination spectrum and at 4–8-fold dilutions the reaction was similar to that of washed bacteria suspended in saline. A similar effect was obtained with 0.1% agar at 10^{-5}, 1% peptone at 10^{-2}–10^{-3}, and 0.1% albumin at 10^{-4} dilution. In the presence of meat extract the agglutination ceased or narrowed in spectrum, depending on the concentration.

Virulent strains failing to agglutinate under routine conditions usually became agglutinable at 10^{-1}–10^{-2} dilution of 0.1% agar in the pH range

1.0–2.3. Avirulent strains, however, remained inagglutinable when examined in this manner. After dialysis the above solutions exerted the same effect.

Virulent *S. flexneri* cultures failed to agglutinate after washing in ethanol or cetylpyridinium bromide. The agglutination spectrum was broadened by a previous treatment with acetone. Washing in chloroform was ineffective or caused an unimportant narrowing of the agglutination spectrum. *S. sonnei*, which showed a wide range agglutination, was not influenced by any of the above treatments.

Cultures subjected to heating at 100 °C for 2 hr behaved as follows. Thirty-two out of 50 virulent *S. flexneri* strains giving typical agglutination lost their agglutinability; the agglutination spectrum narrowed considerably for 8, slightly for 10 strains. Avirulent, inagglutinable *S. flexneri* strains became partly agglutinable after heating. The majority of virulent *S. sonnei* strains showed no change after heating; some strains showed a narrowing of the agglutination spectrum or became inagglutinable. Avirulent pure phase II *S. sonnei strains* were not influenced by heating.

Finally, the effect on acid agglutination of the culture medium used for subculturing the strains was examined. Media varying in N-source yielded different results as to the appearance and incidence of colony variants. The association between virulence and acid agglutination, however, was not influenced by the ingredients of the medium. There was one exception: peptone water yielded cultures negative in the eye test but agglutinating similarly as the virulent strains did. Agar plate subcultures of these cultures were characterized by a great number of dense colonies opalescent in oblique light, that had lost their virulence. If such colonies were present in a sufficient number, the type of agglutination was characteristic of virulent cultures independently of the state of their virulence.

Discussion

Little is known about the factor determining the virulence of shigellae. Studies have been performed on the histopathology of shigella infection [11] and on models for examining virulence [12–15]. Virulence is most reliably and simply tested by SERÉNY's guinea pig eye method [1]. Good results were obtained by GERBER and WATKINS in tissue culture [16] and by KÉTYI in chicken embryos [17]. Several, mostly unsuccessful or contradictory attempts were made at finding markers associated with virulence: serological and biochemical properties [13, 18–23], fimbriae [24], colony morphology [25–27] exoenzymes [28–32], metabolic differences [33–36]. Of the studies of physicochemical characteristics of the cell surface, those of BOGDANOVA [37] are worth mentioning. She found that the microelectrophoretically measured elektrokinetic potential was higher in avirulent than in virulent variants.

The use of acid agglutination associated with surface factors has long been attempted in bacteriology. MICHAELIS [4] was the first to describe that different bacteria agglutinate at characteristic pH values. This finding was utilized in diagnostic bacteriology by several authors [38–42]. SCHOLTENS [43] and BEGUIN and GRABAR [5] applied this method for the differentiation of Vi and R variants of salmonellae, RAUSS *et al.* [44] for following up the phase variation of *S. sonnei*. The association between virulence and acid agglutination of shigellae was studied by AAZAI [2] and NAKAMURA [3]. The correlation was significant according to AAZAI; this finding was not confirmed by NAKAMURA.

Most of NAKAMURA's discrepant results concerned *S. flexneri* 1b and *S. sonnei*. Virulent *S. flexneri* 1b strains failed to agglutinate while avirulent *S. sonnei* cultures gave a positive reaction. He concluded that acid agglutination was characteristic of the serotype rather than the virulence of the organism. The present studies indicated that the proportion of different colony variants in inhomogeneous cultures influenced the result of acid agglutination. The majority of *S. flexneri* 1b and all *S. sonnei* cultures dissociate readily and are inhomogeneous. This fact explains the discrepant results obtained for these organisms. Isolation of different colony types and conjunctival passage experiments in the present study have demonstrated that other *S. flexneri* serotypes may also be inhomogeneous.

The agglutination in acid buffer series of virulent and agglutinable avirulent cultures is usually different in appearance. Washed suspensions of freshly isolated virulent cultures agglutinate in tubes in the middle of the series. Agglutinable avirulent strains show reaction in tubes with low pH buffer or in every tube and form coarse granules which do not disintegrate readily on shaking.

Different factors are probably responsible for the agglutination of virulent and of avirulent cultures. Chemicals and heating are influencing the reaction of the former cultures but are ineffective or exert a kind of adverse effect on the latter ones. Artificial conditions in maintaining the strains evidently alter the physicochemical structure of the cell surface, since the sooner after isolation the strains are tested and the less the laboratory manipulations performed with them, the best the correlation between virulence and acid agglutination.

Although it is improbable that the factor responsible for acid agglutination should directly be associated with the pathomechanism of infection, the method is advantageous for a simple and not laborious examination of a great number of cultures. The results are read after 24–48 hr incubation in contrast to the one week observation of guinea pigs tested for eye reaction. The method is rendered even more reliable if it is connected with the examination of colony morphology.

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VIRULENCE AND ALUMINIUM HYDROXIDE GEL ADSORPTION OF SHIGELLA

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Summary. The proportion of bacterial cells adsorbed to $Al(OH)_3$ gel varies from strain to strain. Adsorption is inhibited by phosphate ions; their concentration allowing adsorption of 50% of the cells has been termed AC_{50}. This value was usually higher for virulent (0.1–0.001 M) than for avirulent (0.00065 M or less) *Shigella flexneri* cultures and was influenced by colony morphology, number of previous subcultures, ingredients of the medium and passage on the guinea pig eye. The AC_{50} was unrelated to the serotype of the strains. In view of strain-to-strain differences in AC_{50}, instead of absolute values the relative difference between derivatives of the strain examined should be considered in order to distinguish virulent and avirulent variants.

An association between virulence of shigellae and the spectrum of agglutination in acid buffer series described in a previous paper [1] pointed to the existence of physico-chemical differences in the cell surface of avirulent and virulent variants. Adsorption experiments on $Al(OH)_3$ gel have shown differences between strains within the same serotype and even the phage type of *Enterobacteriaceae* and revealed that pathogenic *Escherichia coli* strains differ in this respect from apathogenic ones [2]. Inspired by these findings, an attempt was made to distinguish between virulent and avirulent *Shigella flexneri* strains by the use of the method.

Materials and methods

Bacterial strains isolated freshly from patients, avirulent and virulent variants obtained after subculturing, and avirulent strains stored at 5 °C were used. Subcultures were performed on agar plates, in peptone water and in synthetic media containing different N-sources [3]. Colony morphology was examined at oblique transmitted light [4]. Virulence was tested by the guinea pig eye technique [5].

Adsorption experiments. Bacterial suspensions adjusted to standard density were added to $Al(OH)_3$ gel supplemented with a series of 2-fold dilutions of phosphate buffer in the range of 0.33 to 0.00065 M. phosphate ions inhibited the adsorption of bacterial cells proportionally to their concentration. Each strain was characterized by the molarity of phosphate buffer allowing a 50% adsorption of the cells (AC_{50} value).

The density of the supernatant containing the unadsorbed cells was at the beginning estimated by measuring optical density [2]. Later, ^{32}P-labelled bacteria were used. Into an Erlenmeyer flask, 20 ml broth and 0.25 mCi carrierless ^{32}P were measured, then the medium was inoculated with a loopful of culture and incubated at 37 °C overnight. The bacteria were washed 5 times in 20 ml saline adjusted with Tris buffer to pH 7.5, then resuspended in Tris buffer so as to give about 5000 cpm. Other details of the adsorption experiments have been described previously [2, 6].

Results

Table I shows that virulent cultures tend to give higher, while avirulent cultures lower AC_{50} values. A small number of cultures failed to follow this rule. The results were influenced by colony morphology. Virulent cultures with high AC_{50} values consisted purely of translucent, greenish ('G') colonies [4, 7, 8]. Less translucent, reddish ('R') colonies were characterized by lower AC_{50} values (Table II). Avirulent cultures forming opaque colonies after subculturing in peptone water were adsorbed by $Al(OH)_3$ gel as markedly as were the virulent cultures (last right hand column of Table III). The results are influenced by an inhomogeneity of the culture (presence of morphologically different colonies). The number of subcultures may also alter the result: virulent variants isolated after several passages tend to give lower AC_{50} values.

A comparison of pairs of variants isolated from the same strain after the same number of subcultures showed that the virulent form yielded higher AC_{50} values. This is obvious from Table III, which presents the effect on the

Table I

AC_{50} spectrum of virulent and avirulent S. flexneri cultures

AC_{50}	No. of strains	
	Virulent	Avirulent
0.1–0.06	20	7*
0.05–0.001	74	30
0.00065 or less	27	70
–(30–50%)**	6	21

* Two out of the 7 strains were old laboratory cultures stored in the refrigerator; 3 variants isolated from peptone water formed opalescent colonies (last column of Table III); 2 strains of unusual behaviour belonged to serotype 4aA

** In the absence of phosphate ions not more than 30—50% of the cells were adsorbed

Table II

Colony morphology and AC_{50} value of virulent cultures

AC_{50}	Greenish glistening colonies	Reddish colonies
0.1–0.06	20	—
0.05–0.001	58	7
0.00065 or less	11	16
–(30–50%)*	3	3

* In the absence of phosphate ions not more than 30—50% of the cells were adsorbed

Table III

AC_{50} values for virulent and avirulent pairs of S. flexneri strains obtained by subculturing in different media

Serotype	Strain	$NH_4H_2PO_4$		Asparagine		Peptone water		Peptone water, opalescent colonies
		Virulent	Avirulent	Virulent	Avirulent	Virulent	Avirulent	Avirulent
2a	009	0.06–0.10	0.001–0.002	.	.	0.03–0.06	<0.00065	0.004–0.008
2a	244	0.10	0.00065	.	.	0.10	<0.00065	0.008–0.016
2a	377	0.10	0.002–0.004	0.008–0.016	0.001–0.002	0.004	<0.00065	0.03–0.066
2a	473	0.016–0.033	0.004	0.008–0.016	<0.00065	0.008–0.016	.	0.004–0.008
3a	226	0.03–0.06	0.0	0.004	0.0	0.03–0.06	<0.00065	.
3a	275	0.016	0.0	0.016–0.033	<0.00065	0.004–0.008	0.0	0.06–0.10
3a	299	0.06–0.10	<0.00065	0.066	<0.00065	0.016–0.033	<0.00065	0.10
3a	384	0.03	<0.00065	>0.10	0.00065–0.001	>0.10	<0.00065	0.001
3a	613	0.002–0.004	<0.00065	0.008–0.016	<0.00065	0.008–0.016	<0.00065	0.066–0.10
4a	372	0.06–0.10	0.03–0.06	0.06–0.10	0.06–0.10	.	.	.
X var.	717	0.002–0.004	0.00065	0.001	<0.00065	0.001–0.002	0.00065	0.004

AC_{50} value of different media used in subculturing. For strains 275, 299, 613 and 473 the pairs of variants were isolated from the 5th subculture, for other cultures from the 9th to 14th subculture. The parent strains were isolated freshly from patients. Table III shows that adsorption was slightly influenced by the ingredients of the medium and that the difference in AC_{50} between virulent and avirulent bacteria remained evident in every medium. Opalescent avirulent variants grown from peptone water behaved exceptionally in that they yielded AC_{50} values characteristic of virulent cultures. It is also evident that there was no difference in AC_{50} according to serotypes.

Discussion

The present results have revealed the factors influencing the parallelism between virulence and AC_{50} value of *S. flexneri* cultures. In examining acid agglutination it has been revealed that the alteration of virulence may be accompanied with a change in colony morphology and that colony morphology itself may influence the result of acid agglutination. If the factors influencing AC_{50} values (number of subcultures, ingredients of the medium and guinea pig eye passage) can be standardized, the results of the test correspond in reliability to other biological methods. Some cultures are unsuitable for AC_{50} determination due to their flat elution curve or to the fact that the majority of their cells are not adsorbed even in the absence of phosphate ions. A strict technical control of the method is essential, since even slight chemical impurities may considerably influence the adsorption of bacteria [6].

It is not known whether virulence is directly associated with the surface factors determining gel adsorption or there is a mere parallelism between the two properties. In this respect it should be mentioned that pathogenic serogroups of *E. coli* showed a weaker adsorption than apathogenic ones [2].

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IS IT POSSIBLE TO DILUTE OUT THE INDUCER FROM ESCHERICHIA COLI CELLS?

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Summary. The inducer dilution technique is proposed for stopping mRNA synthesis on the *lac* operon of induced *Escherichia coli* cells. Evidence is presented to show that the procedure, *i.e.*, 50-fold dilution of the induced culture in inducer-free medium, does not stop β-galactosidase formation after a longer induction period in wild type, y^+ cells. Dilution in glucose-containing medium strongly inhibits β-galactosidase synthesis by transient repression.

The *Escherichia coli lac* operon proved to be a very useful tool for investigating transcription and translation. These processes can be studied simultaneously but there are improved techniques for separating them, allowing kinetic measurements of either process [1].

To stop mRNA synthesis on the *lac* structural genes, it is sufficient to remove the gratuitous inducer (commonly, isopropyl-β-D-thiogalactoside: IPTG, or methyl-β-D-thiogalactoside: TMG), which allows restitution of the *lac* repressor protein and the *lac* operator complex. The inducer can be removed by filtration of the induced bacterial suspension on membrane filter, then washed and resuspended in pre-warmed, inducer-free medium [2, 3].

This procedure washes out the inducer molecules from the cells and it is effective, although not sufficient for fine kinetic analysis, because the operation needs about one to three minutes [3, 4].

Another alternative is the so-called inducer dilution technique, a 50-fold dilution of the induced culture in pre-warmed inducer-free medium [5, 6]. This washes out the inducer into the medium, which decreases to such a low intracellular concentration as to allow the repression of *lac* structural genes by the *lac* repressor. It was proposed to use 10^{-4} M IPTG, which is five times less than the usual concentration of this inducer in β-galactosidase induction. This method has been applied for the useful pulse induction technique [6], too, which results in accurate fine analysis of the induction and repression kinetic of the *lac* operon [7].

When using the inducer dilution technique, we failed to obtain reliable results with our *E. coli* K-12 strain after longer induction period. This led us to conclude that it is not the dilution of the inducer, but the glucose present in the diluting inducer-free medium in some experiments [7] that may be made responsible for the effect counteracting the induction of the procedure.

Materials and methods

E. coli K-12 wild type cells were used throughout. An overnight culture of the bacteria was diluted in fresh, pre-warmed mineral salts + glycerol medium [8] to an $OD_{570} = 0.075$ and cultivated in a reciprocal shaker (Gallenkamp IH-350) at 37 °C. At $OD_{570} = 0.300$, *i.e.* in the log phase, a part of the cell suspension was induced with 5.10^{-4} M and another aliquot with 10^{-4} M IPTG (Mann Research Laboratories): 0 min. Twenty or 30 min after the induction, 0.2 ml aliquots of the induced cultures were diluted in 10.0 ml of the same, pre-warmed medium lacking IPTG, without or with 10^{-3} M glucose. (Practically 51-fold dilution.) From these diluted suspensions, 0.2 ml samples were taken and pipetted into test tubes containing 50 μg chloramphenicol in 0.1 ml pH 7.0 phosphate buffer. These were kept in an ice bath until the estimation of β-galactosidase activity. First the samples were toluenized at 37 °C and then incubated with o-nitrophenyl-β-D-galactopyranoside (ONPG, Fluka) at 37 °C for 1 hr. The reaction was stopped by adding 1.0 ml of 1.0 M Na_2CO_3. After centrifugation, the absorbance of the supernatants was read at 420 nm in a UNICAM SP 1800 spectrophotometer. The enzyme activity was expressed as mμ mole ONPG hydrolized per hour.

The experiments were repeated eight times, also using a glucose concentration of 10^{-2} M. In other experiments, dilution was made 40 s after induction with crystalline IPTG (final concentration 10^{-4} M).

Results

As can be seen in Fig. 1, a 50-fold dilution in inducer-free medium did not result in complete stopping of induced β-galactosidase synthesis, in the presence of neither $5 \cdot 10^{-4}$ M nor of 10^{-4} M IPTG. After dilution there was a transient decrease in the rate of enzyme synthesis. At higher inducer concentration, the rate of β-galactosidase production was somewhat higher. Later,

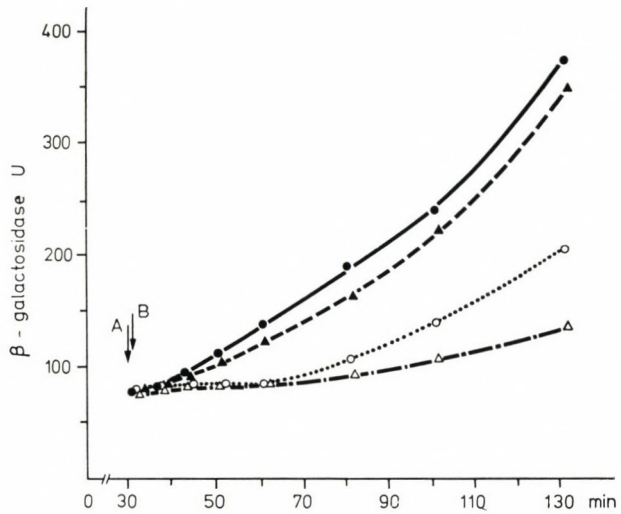

Fig. 1. E. coli K-12 wild type strain. Induction with IPTG at 0 minute; 30 and 31 min after the induction (arrows A and B, respectively), 0.2 ml aliquots of the induced cultures were diluted in 10.0 ml of the same, pre-warmed medium lacking IPTG, without or with 10^{-3} M glucose. ●——● 5.10^{-4} M IPTG, dilution in glucose-free medium; ○·····○ 5.10^{-4} IPTG, dilution in glucose-containing medium; ▲– – –▲ 10^{-4} M IPTG, dilution in glucose-free medium; △-·-·-·-△ 10^{-4} M IPTG, dilution in glucose-containing medium

the rate of enzyme synthesis increased somewhat. In contrast, dilution in glucose-containing medium resulted in a complete inhibition of β-galactosidase synthesis, which lasted for about 25 min when applying $5 \cdot 10^{-4}$ M IPTG, and for about 35 min with 10^{-4} M IPTG. After this period, enzyme synthesis started again, the case of a higher inducer concentration at a higher, and with lower inducer doses at a very low rate. This was typical for glucose-repression; formation of β-galactosidase stopped for a while (severe transient repression) and then it started again at a rate lower than in the control (permanent catabolite repression).

Discussion

KEPES [7] interpreted the decrease and later the cessation of β-galactosidase synthesis as a consequence of the lack of effective inducer from the cells to the inducer-free medium. This would stop mRNA-polymerase movement along the *lac* operon, thus an exhaustion of the *lac* mRNA pool in the cell would lead to decreased β-galactosidase synthesis, which levels off about 6—8 min later. He used the amount of β-galactosidase synthetized after the dilution as a measure of the amount of *lac* mRNA present at the time of dilution, and followed enzyme synthesis for 18 min after dilution, assuming that this time was enough for the exhaustion of enzyme-forming capacity based on previously synthetized and continuously degrading mRNA molecules.

Application of the inducer dilution technique using y^+ cells after a longer induction period needs glucose-containing diluting medium, and in this case the point of attack is not the *lac* operator, but the *lac* promoter region. It is well-known that glucose, added to induced cultures of *E. coli* growing on non-glucose carbon source (*e.g.* glycerol, succinate, etc.), evokes a short, absolute or nearly absolute inhibition of β-galactosidase synthesis: the so-called transient repression, which is followed by a less severe, the so-called catabolite (or permanent) repression. Studies of these phenomena have shown that the transient and catabolite repressions are mediated through cyclic adenosine-3',5' monophosphate (cAMP) and cAMP receptor protein (CRP, [9]). JACQUET and KEPES [1] have shown that the glucose repression and its counteraction by cAMP are mediated at the level of transcription.

It has therefore been concluded that the inducer dilution technique with a longer induction period can be applied as described above and actually causes a transient severe repression and prevention of mRNA synthesis (*i.e.* transcription of the *lac* DNA) due to the glucose present in the diluting medium and does not act upon the *lac* operator, but upon the *lac* promoter. The outcome is the same, except that the cessation of transcription by repression may be somewhat more instantaneous than the one which would have been caused by the inhibition of initiation.

Although our results do not affect the reliability of previous experimental data, substances which are to be tested and which affect transient repression may lead to erroneous explanations concerning the transcription and translation of *lac* operon when using the inducer dilution technique.

In the case of pulse induction, the dilution in inducer and glucose-free medium resulted in a complete block of β-galactosidase synthesis for about 35–40 min, but later enzyme synthesis started again. In the presence of 10^{-2} M glucose in the diluting medium, the lag was about 80 min. This finding and the fact that KEPES [7] used mostly y^- cells, point to the role of galactoside permease, the product of *lac y* gene. The level of this enzyme must be much lower after a short pulse induction, but it still seems sufficient for accumulating the diluted inducer in the cells after a longer period.

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METRONIDAZOLE RESISTANCE, A GENUS-SPECIFIC FEATURE OF LEPTOSPIRES

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Summary. Parasitic and saprophytic leptospires are highly resistant to metronidazole. Their growth and motility was not inhibited by 100 μg/ml metronidazole either under aerobic. or under anaerobic conditions. Metronidazole resistance seems to be a genus-specific feature of the leptospires, which differentiates them from the borreliae and treponemes.

For the differentiation of spirochaetes there are few methods and some of them cannot be easily applied in practice, since several strains are difficult to maintain and certain species are not cultivable at all.

Metronidazole is in wide use for the treatment of genital trichomoniasis and amoebiasis. The drug has recently been found to be active against spirochaetes and different species of borrelia and treponema have been reported to be susceptible to metronidazole [1–6]. In previous studies we have, however observed that some leptospira strains tested were resistant to metronidazole [7, 8.] On the basis of these findings we have decided to test the *in vitro* effect of the drug on the growth and motility of a number of leptospira strains in order to elucidate whether the metronidazole resistance was a characteristic feature of leptospires which might be useful for differentiating the genera in the family *Treponemataceae*. The present paper discusses the results of these investigations.

Materials and methods

Organisms. Seventy-five parasitic and 15 saprophytic strains were tested. The parasitic leptospires represented 13 serogroups and 47 serotypes: *Leptospira icterohaemorrhagiae, mankarso, naam, birkini, smithi, ndambari, budapest, javanica, poi, coxi, canicola, jonsis, sumneri, schueffneri, malaya, pyrogenes, zanoni, abramis, biggis, hamptoni, butembo, cynopteri, bangkinang, erinacei-auriti, mooris, djasiman, gurungi, australis, jalna, fugis, bratislava, pomona, grippotyphosa, hebdomadis, kremastos, worsfoldi, kabura, szwajizak, hardjo, medanensis, wolffi, sejroe, saxkoebing, ricardi, bataviae, paidjan, tarassovi.*

Of the saprophytic complex the following strains were used: AM-3, AM-6, AM-8, AM-12, AM-20, AM-21, USA free living, Gand IV, WaZ, Tiburtino, Patoc 1, Ancona, Leeds, Semaranga and Andaman A.

All the strains were maintained in Korthof's medium (10% heat inactivated rabbit serum with added haemoglobin, pH 7.2) and were transferred at 2-week intervals before testing.

Growth inhibition was studied in liquid Korthof's medium. Metronidazole (Klion, Richter, Budapest) was dissolved in hot distilled water and added to the medium at 1, 10 and

100 μg/ml concentrations. Then 2 ml amounts of the medium were dispensed aseptically in tubes and these were closed by rubber plugs. The tubes were inoculated with 0.2 ml of 7–10-day-old cultures and visible growth was read after 10 days incubation at 30 °C. In further experiments, representative strains of each serogroup were tested in semisolid Korthof's medium (0.1% agar), in liquid Korthof's medium containing 0.1% sodium thioglycollate, and in liquid Korthof's medium supplemented with thiamine hydrochloride (10 μg/ml), vitamin B_{12} (1 μg/ml) and yeast extract (1%).

The effect of metronidazole on the motility of parasitic and saprophytic leptospires was studied by dark-field microscopy of cultures in liquid Korthof's medium and liquid Korthof's medium with 0.1% sodium thioglycollate, each containing 100 μg/ml metronidazole. To 1.8 ml of the media, 0.2 ml of 3–4-day-old cultures were added and the tubes were incubated aerobically at room temperature and at 30 °C for 20 hr. Then strains representing different serogroups of parasitic leptospires were examined at 35 °C by using the above technique, furthermore after incubation anaerobically at 30 °C and 35 °C. Percentage motility was determined by counting 100 cells of each tube under dry ×400 magnification and compared to drug-free controls.

Results

Metronidazole at the concentrations employed failed to affect the growth of leptospires. All the 75 parasitic strains and the 15 saprophytic strains showed uninhibited development at 100μg/ml of metronidazole in liquid and semisolid Korthof's medium. Neither was the lag-phase prolonged by the drug. There were no differences in this respect between old and recently isolated strains of various serotypes or among strains cultured from man, animals or surface waters in several continents. Addition of sodium thioglycollate to the medium did not influence the results and the same resistance was observed in Korthof's medium supplemented with thiamine hydrochloride (10 μg/ml), vitamin B_{12} (1 μg/ml) and yeast extract (1%).

Metronidazole proved to be ineffective against leptospires also in motility experiments. Neither loss nor partial inhibition of motility of parasitic leptospires exposed to 100 μg/ml of metronidazole could be demonstrated in liquid Korthof's medium and in Korthof's medium containing 0.1% sodium thioglycollate at room temperature, 30 °C and 35 °C, within 20 hr. Under anaerobic conditions, suspensions of parasitic leptospires showed the same motility in the presence of 100 μg/ml of metronidazole as did the drug-free controls after incubation at 30 °C and 35 °C for 20 hr. Saprophytic leptospires tested at room temperature and at 30 °C showed normal motility patterns at 100 μg/ml.

Discussion

The antispirochaetal activity of metronidazole was first suspected when SHINN observed the effectiveness of the drug in Vincent's stomatitis [9]. DAVIES *et al.* [1] reported that oral spirochaetes obtained from scrapings of gingivitis cases were immobilized in the presence of 4 μg/ml of metronidazole within 15 min and *Treponema reiteri* required 0.02 μg/ml for inhibition of growth. Then various

species of treponemes and borreliae were tested for susceptibility to the compound, and all cultures were inhibited or immobilized at a concentration of 10 μg/ml or less. DAVIES [3] showed that metronidazole was effective against *Treponema pallidum in vivo* and high doses of the drug caused a disappearance of treponemes from lesions of human secondary syphilis. WILKINSON *et al.* [4] noted a reduction in the number of spirochaetes in lesions of early syphilis during metronidazole therapy. The concentration of metronidazole causing 50% immobilization of treponemes in TPI medium was 5.2 μg/ml in 18 hr at 35 °C under anaerobic conditions. LE CLAIR and KEETIN [5] studied the *in vitro* immobilizing action of the compound on different oral spirochaetes, *T. reiteri* and *T. pallidum*. The cultures of oral spirochaetes and *T. reiteri* were tested in thioglycollate medium containing 0.1% agar and 10% rabbit serum under aerobic conditions. *T. pallidum* was examined in inactivated rabbit serum diluted with buffered saline and kept in 95% nitrogen plus 5% carbon dioxide atmosphere. All tubes were incubated at 35 °C for 24 hr. Metronidazole immobilized all the spirochaetes tested and the following mean concentrations produced 50% immobilization of the different species: *Borrelia buccalis*, 6.25 μg/ml; *Borrelia vincentii*, 5.20–6.25 μg/ml; *Treponema microdentium* and small oral treponemes, 3.06–4.68 μg/ml; *T. reiteri*, 0.42 μg/ml; and *T. pallidum*, 5.20 μg/ml. HOLM and MOBACKEN [6] reported that *T. pallidum* suspensions were partially immobilized at 10 μg/ml and almost completely immobilized at 100 μg/ml of metronidazole in TPI medium at 35 °C in 18 hr under anaerobic conditions.

The present results have confirmed our previous finding on the metronidazole resistance of leptospires and indicate that it is a characteristic feature of the genus *Leptospira*. Testing a high number of parasitic and saprophytic strains we could not demonstrate any inhibitory action of 100 μg/ml of metronidazole on their growth and motility. They displayed the same resistance in different media containing rabbit serum, agar, or sodium thioglycollate and their motility was not affected by the compound under anaerobic conditions. Since borreliae and treponemes have been found susceptible to the drug under similar conditions, the above data clearly show the difference in the action of metronidazole against aerobic and anaerobic spirochaetes. These observations are consistent with the results of our former studies on the antibacterial activity of metronidazole, which have suggested that this nitroimidazole compound may be regarded as a selective inhibitor of anaerobic microorganisms [7, 8, 10–16].

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DOUBLE GEL DIFFUSION STUDIES ON PSEUDOMONAS AERUGINOSA O ANTIGENS

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Summary. *Pseudomonas aeruginosa* antigen type strains of different authors and *Enterobacteriaceae* O antigens related to *P. aeruginosa* were tested by double agar gel diffusion against unabsorbed and absorbed O immune sera. From LÁNYI's 23 O antigen type strains three different extracts were prepared: (a) supernatant of agar cultures suspended in saline and steamed for 2 ½ hr; (b) L_1 fractions and (c) purified LPS fractions of Westphal phenol water extracts. In homologous antigen–antibody systems the number of precipitation lines varied not only with the strain tested but also with the kind of extract; usually, L_1 fractions gave the highest number of lines. Cross-precipitation with saline and LPS extracts reflected, with the exception of non-precipitating partial antigens O5d and O7c, the same group and subgroup specific antigens as demonstrated by agglutination, while L_1 fractions of certain type strains (O7a,7b, O7a,7c, O8, O9, O11 and O13) failed to react. Results with saline extracts of 47 other *P. aeruginosa* antigen type strains (HABS, FISHER, LÁNYI) and of 3 *Enterobacteriaceae* serogroups sharing major O antigens with *P. aeruginosa* also corresponded to the antigenic structure determined by agglutination. Minor somatic factors demonstrated by agglutination of cultures heated at 130 °C were not detectable with the double diffusion technique used.

In a study of the O antigens of *Pseudomonas aeruginosa*, LÁNYI [1] demonstrated two kinds of somatic factor. In addition to group specific antigens practically all *P. aeruginosa* strains contain a common thermostable component. The latter produces low titre antibodies detectable with heated but not with living bacteria. The studies of ÁDÁM *et al.* [2] on the serological behaviour of *P. aeruginosa* lipopolysaccharides revealed that the common factor can be detected with LPS-coated erythrocytes but not by tube precipitation test. These findings inspired us to employ the double diffusion technique for a further investigation of *P. aeruginosa* antigens.

Materials and methods

Bacterial strains. P. aeruginosa type strains of LÁNYI's [1, 3], HABS' [4] and FISHER's [5] antigenic schemata were used.

Saline extract. Peptone-meat extract agar cultures grown for 20 hr at 37 °C were suspended in merthiolate-saline (merthiolate, 0.1 g; NaCl, 8.5 g; distilled water, 1000 ml) and heated at 100 °C for 2 ½ hr. After centrifugation at 4000 rpm the supernatant was used. Each ml of the extract represented approximately 12 mg bacteria (moist weight).

L_1 extract was prepared by WESTPHAL and JANN's phenol water method [6] as employed by ÁDÁM *et al.* [2]. The crude extract was dialyzed, freeze-dried, rehydrated and ultracentrifuged at 105 000 *g*. The supernatant (L_1) was freeze-dried and stored. Stock solutions were prepared by dissolving 5 mg L_1 in 1 ml merthiolate-saline and heating the solution at 100 °C for 1 hr.

Purified LPS extract was prepared as described in reference [2]. To make a stock solution, 5 mg LPS were dissolved in 1 ml merthiolate-saline and heated at 100 °C for 1 hr.

Immune sera. Rabbits were immunized with LÁNYI's O antigen type strains heated at 75 °C for 1 hr [1]. The sera were preserved with phenol at 0.5% final concentration. Serum absorption for gel diffusion test was performed as described for agglutination test [1], except that the sera were absorbed undiluted.

Agar gel. Agar No. 1 (Oxoid, London), 8.5 g; K_2HPO_4, 9.2 g; KH_2PO_4, 1.8 g; NaCl, 6.0 g; merthiolate, 0.1 g; distilled water, 1000 ml. After dissolving the ingredients, the hot medium was stirred for 5 min with an Ultraturrax apparatus, distributed into bottles at 50 ml amounts and steamed without pressure for 1 hr.

Agar diffusion technique. Onto clean slides 25 × 75 mm in size, 3.5 ml agar gel were poured, then holes 3 mm in diameter were made by means of a cutter so that six peripheral wells were arranged hexagonally around a central well. The distance between the centres of each pair of wells was 9 mm. Into the wells 10 μl antigen solution or immune serum were pipetted. The slides were incubated in a moist chamber at 37 °C for 3 days. After washing in saline, staining of the preparations was performed with amidoblack [7].

Results

1. *Antigen–antibody reactions obtained with different extracts.* In the first part of the experiments undiluted immune sera were pipetted into the central wells. The peripheral wells were filled with two-fold serial dilutions (1 : 1–1 : 128) of saline extracts, L_1 fractions and LPS fractions of the homologous strains.

The majority of sera qualified good on the basis of tube agglutination titre and stored not longer than for 1 year, gave precipitation lines after 24 hr with the homologous antigens diluted 1 : 16 or above. As a rule, L_1 extracts reacted at the highest dilution, LPS extracts were somewhat less reactive and saline extracts gave a considerably lower titre. After an incubation lasting longer than 24 hr, the lines became more intensive and appeared at higher antigen dilutions. The number of lines obtained in a given antigen–antibody system varied not only from strain to strain, and with the quality of the serum, but depended highly on the kind of extract used. L_1 fractions of practically every strain gave 2 or 3, sometimes even 4 or 5 lines after 72 hr (non-reacting L_1 fractions will be dealt with later in this paper). LPS antigens and especially saline extracts gave less lines: LPS and saline extracts for 7 and 10 different type strains, respectively, showed only one line with the sera used. Fig. 1 presents an example of this finding. The tendency of L_1 extracts to give the highest number of lines is evident. The number of lines seems to vary mainly with the quality of the extract and less with its antigen concentration (the single line obtained with saline extract is as marked as the corresponding lines with L_1 and LPS fractions).

In the second part of the experiments serial dilutions of the immune sera were measured into the peripheral wells and tested for reaction with 1 : 1 to 1 : 8 dilutions of the homologous saline extracts and L_1 antigens pipetted into the central well. Fig. 2 shows that the intensity of lines decreased parallel

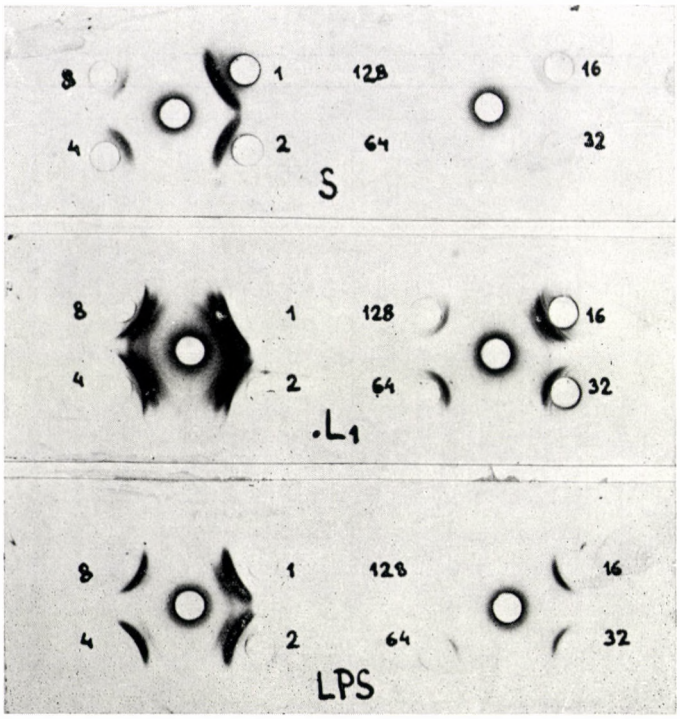

Fig. 1. Precipitation reactions of twofold serial dilutions of saline, L_1 and LPS extracts (strain 170005) against the homologous undiluted serum. Central well: serum; peripheral wells: antigen (the figures stand for the reciprocals of antigen dilutions); S = saline extract; L_1 = L_1 fraction; LPS = lipopolysaccharide fraction

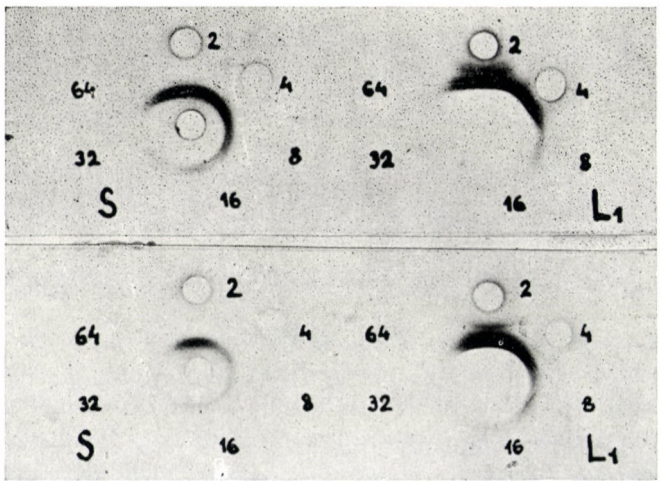

Fig. 2. Precipitation reactions of saline extracts (S) and L_1 fraction (L_1) of strain 170005 against twofold serial dilutions of the homologous serum. Central wells: S and L_1 antigens (upper slide: undiluted; lower slide: diluted 1 : 4). Peripheral wells: serum (the figures stand for the reciprocals of serum dilutions)

Table I

Agar gel cross-reactions of saline, L_1 and LPS antigen extracts of P. aeruginosa type strains

O antigen	Type strain	Unabsorbed									
		1	2	3a,3b	3c	3a,3d	3a,3d,3e	3d,3f	4a,4b	4a,4c	4a,4d
1	170001	+	—	—	—	—	—	—	—	—	—
2	170002	—	+	—	—	—	—	—	—	—	—
3a,3b	170003	—	—	+	±	+	—	±	—	—	—
3c	170004	—	—	±	+	×	±	—	—	—	—
3a,3d	170005	—	—	+	+	+	±	+	—	—	—
3a,3d,3e	170006	—	—	±	—	±	+	+	—	—	—
3d,3f	170007	—	—	+	±	+	+	+	—	—	×
4a,4b	170008	—	—	—	—	—	—	—	+	+	±
4a,4c	170009	—	—	—	—	—	—	—	+	+	±
4a,4d	170010	—	—	—	—	—	—	—	±	+	+
5a,5b,5c	170011	—	—	—	—	—	—	—	—	—	—
5a,5b,5d	170012	—	—	—	—	—	—	—	—	—	—
5a,5d	170013	—	—	—	—	—	—	—	—	—	—
6	170014	—	—	—	—	—	—	—	—	—	—
7a,7b	170015	—	—	—	—	—	—	—	—	—	—
7a,7c	170016	—	—	—	—	—	—	—	—	—	—
8	170017	—	—	—	—	—	—	—	—	—	—
9	170018	—	—	—	—	—	—	—	—	—	—
10a	170019	—	—	—	—	—	—	—	—	—	—
10a,10b	170020	—	—	—	—	—	—	—	×	×	×
11	170021	—	—	—	—	—	—	—	—	—	—
12	170022	—	—	—	—	—	—	—	—	—	—
13	170023	—	—	—	—	—	—	—	—	—	—

\+ Precipitation line(s) after 24 hr
± Precipitation line(s) after 48—72 hr

with the dilution of the serum. With undiluted antigens the lines fell nearer to the serum reservoir than with diluted antigens. From the distance of the lines to the serum well it is evident that the L_1 fraction contained the antigen(s) at a higher concentration than the saline extract.

2. *Comparison of cross-precipitation patterns with the antigenic schema established on the basis of agglutination.* Unabsorbed immune sera representing the 23 O groups and subgroups were pipetted undiluted into the central wells. Stock solutions of saline, L_1 and LPS antigens prepared from the corresponding

O sera													
5a,5b,5c	5a,5b,5d	5a,5d	6	7a,7b	7a,7c	8	9	10a	10a,10b	11	12	13	Positive reactions in absorbed sera
−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	×	−	−	−	−	−	−	−	3b+
−	−	−	−	−	×	−	−	−	−	−	−	−	3c+
−	−	−	−	−	−	−	−	−	−	−	−	−	3d+
−	−	−	−	−	−	−	−	−	−	−	−	−	3d+,3e+
−	−	−	−	−	−	−	−	−	−	−	−	−	3d+,3f+
−	−	−	−	−	−	−	−	−	−	−	−	−	4b±
−	−	−	−	−	−	−	−	−	−	−	−	−	4c+
−	−	−	−	−	−	−	−	−	−	−	−	−	4d+
+	+	+	−	−	−	−	−	−	−	−	−	−	5b+,5c+
±	+	+	−	−	−	−	−	−	−	−	−	−	5b+
±	+	+	−	−	−	−	−	−	−	−	−	−	−
−	−	−	+	−	−	−	−	−	−	−	−	−	−
−	−	−	−	+*	+*	−	−	−	−	−	−	−	7b±*
−	−	−	−	+*	+*	−	−	−	−	−	−	−	−
−	−	−	−	−	−	+*	+*	−	−	−	−	−	−
−	−	−	−	−	−	−	+*	−	−	−	−	−	−
−	−	−	−	−	−	−	−	+	+	−	−	−	−
−	−	−	−	−	−	−	−	±	+	−	−	−	10b+
−	−	−	−	−	−	−	−	−	−	+*	−	−	−
−	−	−	−	−	−	−	−	−	−	−	+	−	−
−	−	−	−	−	−	−	−	−	−	−	−	+*	−

× Faint line after 48—72 hr with LPS antigen only
+* No precipitation with L_1 fractions

type strains were measured undiluted into the peripheral wells. Cross-precipitation patterns obtained in this manner are shown in Table I.

Double agar diffusion with saline extracts and with LPS antigens reflected, except for partial antigens 5d and 7c, in every detail the antigenic structure demonstrated by agglutination [1]. With L_1 fractions the majority of reactions corresponded to bacterial agglutination. L_1 fractions of antigens O7a,7b, O7a,7c, O8, O9, O11 and O13 failed to precipitate. As alkali treatment usually improved the reactivity of L_1 antigens, these L_1 fractions were subjected

to heating with 0.25 N NaOH partly at 56 °C, partly at 100 °C for 1 hr, then neutralized with HCl. These treatments were ineffective. Saline extracts of strains 170017 (O8) and 170018 (O9) differed in behaviour from the others in not reacting with the homologous sera. When the saline extracts were prepared with dense suspensions washed off blood agar cultures grown at 37 °C for 1 day then at room temperature for 4 days, a fairly good precipitation was obtained for these strains.

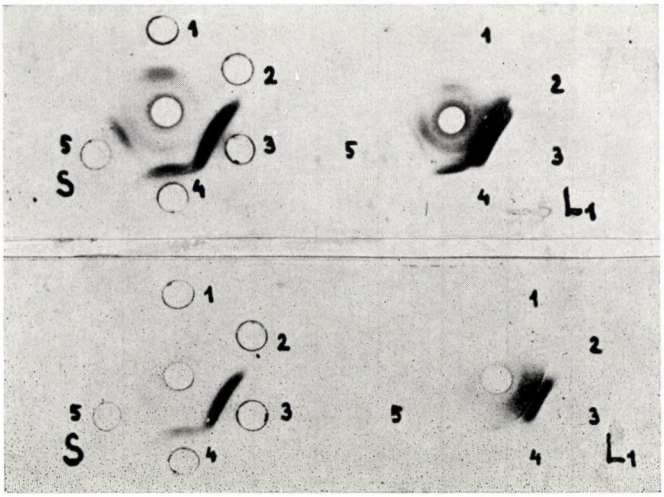

Fig. 3. Upper slide: precipitation lines obtained with undiluted S and L_1 extracts for antigens O3a,3b (1), O3c (2), O3a,3d (3), O3a,3d,3e (4) and O3d,3f (5) against unabsorbed serum O3a,3d (central well). Lower slide: reactions of the same antigens against serum O3d (serum O3a,3d absorbed with antigen O3a,3b)

With certain batches of sera, cross-precipitations not conforming to the antigenic structure were recorded. These reactions appeared as faint lines after 48–72 hr and were usually demonstrable by agglutination. They were absent with other batches of sera for the corresponding antigen and were, therefore, regarded as aspecific reactions and omitted from Table I. LPS antigen tended to give more aspecific reactions than the other two kinds of extract: in the first series of cross-precipitation experiments (consisting of 529 antigen–antibody systems for each of three kinds of extract), the number of aspecific reactions was 19 for LPS, 6 for saline and 2 for L_1 antigens.

3. *Demonstration of partial (subgroup) antigens by double diffusion technique.* In these experiments undiluted sera for the type strains were absorbed with cultures recommended for the preparation of subgroup typing sera [1]. The results are shown in the right hand column of Table I. Apart from the unusual behaviour of antigens O5d and O7c, the result of gel precipitation was identical with that of agglutination. Antigens O5d and O7c failed

to precipitate in several batches of absorbed sera (serum O5a,5b,5d absorbed by O5a,5b,5c and serum O7a,7c absorbed by O7a,7b), which gave agglutination titres for antigens O5d and O7c high enough to expect a marked precipitation. When serum O5d was prepared in a different manner (serum O5a,5d absorbed by O5a,5b,5c), not specified for the definition of antigen O5d, extracts of O5d strains gave a faint precipitation line. For agar gel precipitation the technique of absorption was somewhat modified as far as undiluted sera were absorbed with less bacteria than the amount estimated for the preparation of agglutinating sera. Agglutinins to be absorbed were not completely removed in this manner, but for gel precipitation the sera were specific. An example of absorption is presented in Fig. 3.

The presence of antibody O3d in the absorbed serum is evident. The marked line obtained with the homologous system as compared to the faint lines for antigens O3a,3d,3e and O3d,3f indicates that antigen O3d is more prominent in strain O3a,3d than in the other two strains.

4. *Determination of the antigenic structure of other strains with gel diffusion technique.* Unabsorbed and absorbed sera used in the preceding experiments were pipetted into the central wells and undiluted saline extracts were measured into the peripheral wells.

Results shown in Table II indicate that, except for antigens O5d and O7c, there is a complete agreement between grouping by agglutination and grouping by gel diffusion. It should be mentioned that for other authors' strains the data express only the antigenic structure as determined by LÁNYI's sera. As no cross-reactions and cross-absorptions were performed for these strains, they may contain additional antigens not described in LÁNYI's schema.

Many *Enterobacteriaceae* serogroups shown by a sensitive technique to share parts of somatic antigens with *P. aeruginosa* [8, 9] failed to react when tested by gel diffusion. Major antigenic relationships were, however, demonstrable with this method as well as by slide and tube agglutination.

Discussion

VAN DEN ENDE [10] showed that according to tube precipitation with *P. aeruginosa* Boivin extracts the isolates fell into the same 6 antigenic groups as by agglutination test with alcoholized bacteria. In examining the hydrochloric acid and formamide extracts of *P. aeruginosa*, KÖHLER [11] established 8 serological groups by tube precipitation test. KLEINMAIER and MÜLLER [12] demonstrated that tube precipitation with different *P. aeruginosa* extracts reflected the same antigenic pattern as that obtained by tube agglutination and that intergroup reactions were unfrequent. With double agar gel diffusion test MÜLLER and KLEINMAIER [13] showed that in *P. aeruginosa* antigen–

Table II

Agar gel reactions of saline extracts of P. aeruginosa and serologically related Enterobacteriaceae cultures in P. aeruginosa sera

Strains	Somatic antigens*	Agar gel reaction in unabsorbed and absorbed *P. aeruginosa* sera
171031, 171059, HABS 3	1	1+
171074, 171076, HABS 10, FISHER 5	2	2+
171092	3a,3b	3a,3b+ 3c± 3a,3d+ 3b+
171124, HABS 2, FISHER 3	(3a),3c	3a,3b+ 3c+ 3a,3d,3e+ 3c(abs.)+
171175, HABS 5, FISHER 7	3a,3d	3a,3b± 3a,3d+ 3a,3d,3e+ 3d,3f+ 3d+
171188	(3a),3d,3e	3a,3d+ 3a,3d,3e+ 3d± 3e±
171192	(3a),3d,3f	3a,3b+ 3a,3d± 3a,3d,3e± 3d,3f+ 3d± 3f±
171289	4a,4b	4a,4b+ 4a,4c+ 4a,4d± 4b±
171269, 171248	4a,4c	4a,4b+ 4a,4c+ 4a,4d± 4c±
171272	4a,4d	4a,4b± 4a,4c+ 4a,4d+ 4d+
HABS 6, FISHER 1	4 complex	4a,4c+
Escherichia coli O26	(4 complex)	4a,4d±
171304	5a,5b,5c	5a,5b,5c+ 5a,5b,5d+ 5a,5d+ 5b+ 5c+
171312, HABS 8	5a,5b,5d	5a,5b,5c+ 5a,5b,5d+ 5a,5d+ 5b+
171338, 171348, FISHER 6	5a,5d	5a,5b,5c+ 5a,5b,5d+ 5a,5d+
HABS 7	(5 complex)	—
171350, 171365, HABS 1, FISHER 4	6	6+
171401, 171420, HABS 11, FISHER 2	7a,7b	7a,7b+ 7a,7c+ 7b±
171445, 171447	7a,7c	7a,7b+ 7a,7c+
Arizona O19	(7 complex)	7a,7c±
171455, 171464, HABS 9	10a	10a+ 10a,10b+
171478	10a,10b	10a± 10a,10b+ 10b+
Shigella boydii 7	(10a),(10b)	10a± 10a,10b+
171483, HABS 4	11	11+
171518	12	12+
HABS 12	13	13+

* Determined by agglutination in LÁNYI's unabsorbed and absorbed *P. aeruginosa* sera
\+ Precipitation line(s) after 24 hr
± Precipitation line(s) after 48—72 hr
Figures in brackets indicate that the antigen is not identical with the corresponding factor in the type strain

antibody systems 1 to 4 lines developed during 30 days incubation. In *Pseudomonas tabaci* LOVREKOVICH and KLEMENT [14] failed to show by double diffusion more than one precipitation line. HOBBS *et al.* [15] demonstrated

by gel diffusion technique several different antigens in pseudomonads and related bacteria. At least one of the most frequent 6 components was present in all isolates. VAN EEDEN [16] using concentrated trichloroacetic acid extracts of *P. aeruginosa* and OH antisera showed that strains belonging to VAN DEN ENDE's groups produced in double diffusion test a maximum of 5 lines, one of these being common to 7 of the 8 strains examined. Recently, CHESTER *et al.* [17] have shown that lipopolysaccharides prepared from HABS's type strains gave specific reactions against the homologous sera in capillary, ring and double diffusion precipitation tests. They observed a certain amount of cross-reaction between types 2 and 5. This relationship was demonstrated by VÉRON [18] and by BERGAN [19] and has been confirmed in the present study.

Our experiments have also shown that the double gel diffusion technique is suitable for the demonstration of group and subgroup specific antigens in *P. aeruginosa* and is equivalent in reliability with slide or tube agglutination. Since the method is more laborious than slide agglutination, it will hardly be applied in routine typing.

Common intraspecies antigens detectable by tube agglutination with bacteria heated at high temperature or with LPS-coated erythrocytes [2] could not be demonstrated with gel diffusion. Minor antigenic relationships between *P. aeruginosa* and *Enterobacteriaceae* likewise failed to appear with this method. The obvious reason for these findings is that antibodies against these factors are low in titre and conditions were not sensitive enough to demonstrate their presence. Our results cannot be compared with those of other authors [15, 16] demonstrating common antigens, since in the present study extracts containing only somatic factors reacted against sera prepared with heated antigens.

The findings stating that LPS was responsible for O group specificity [2, 17] have been confirmed. However, supernatants of ultracentrifuged phenol water extracts (L_1 fractions) of certain strains failed to precipitate with the homologous sera. As the corresponding LPS preparations reacted well, it may be assumed that antigens of these strains differed in structure from other *P. aeruginosa* antigens. Whether or not this finding indicates the presence of R-like factors, needs further investigation. The presence of R-like factors as main antigens in group O8 (strain 170017) and in group O9 (strain 170018) seems very probable partly from the findings of ÁDÁM *et al.* [2] and partly from the present studies. It is interesting that the LPS antigens of some of the cultures characterized by non-reacting L_1 fraction differed from LPS antigens of other strains in containing a substance with an absorption peak at 260 nm, that could be removed only by centrifugation at 12 000 *g*.

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